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LIII. THE ABSORPTION AND TRANSLOCATION OF LEAD BY PLANTS.

A CONTRIBUTION TO THE APPLICATION OF THE METHOD OF RADIOACTIVE INDICATORS IN THE INVESTIGATION OF THE CHANGE OF SUBSTANCE IN PLANTS.

By GEORGE HEVESY.

From the Institute of Plant Physiology of the Agricultural High School, and Institute of Theoretical Physics of the University, Copenhagen.

(*Received May 4th, 1923.*)

The investigation of the absorption of lead by plants can be carried out quite simply by dipping them into a solution which contains a radioactive isotope of lead, and determining the radioactivity of the ash from various parts of the plant. In addition to its simplicity and the extraordinary rapidity with which the work can be carried out this method possesses the following advantages:—(*a*) By mixing suitable amounts of ordinary lead with the radioactive lead isotope, one can vary the lead concentration of the solution, as it were, between very wide limits. The assimilation of lead from a $N/1$ solution can be just as readily investigated as that from a solution many million times more dilute. (*b*) One can follow the change in localisation of the lead taken up by the plant, and thence draw conclusions as to the nature of its combination.

The experiments described in this paper were so carried out, that the plants, which had been cultivated in a culture solution, were washed with distilled water, and then the roots were immersed from 1 to 48 hours in a solution containing a mixture of lead nitrate and thorium *B* nitrate. In most cases *Vicia Faba* (horse-bean) was used. After this period of immersion the individual parts of the plant were first well rinsed with distilled water, and then ignited, and the intensity of the radioactivity of the ash was determined by means of an electroscope. This latter magnitude gives directly the lead content of the ash and thus also that of the corresponding part of the plant, when we know the radioactivity and the lead content of the solution in which the plant has been immersed.

Thorium *B* is a transformation product of thorium emanation, and is obtained in a very simple manner. A piece of platinum foil is charged nega-

tively to a potential of 110 volts, say, and suspended in a vessel containing the preparation (radio-thorium, thorium *X*, etc.) from which the thorium emanation is generated. Under these circumstances the thorium *B* collects on the platinum surface, and can be removed with the aid of a few drops of dilute nitric acid. The normality as regards lead of a solution (thorium *B* is an isotope of lead, *i.e.* a substance showing completely the chemical properties of lead) prepared in this way is about 10^{-12}, and if we wish to increase it we only need add to the solution a known amount of lead nitrate. For example, if we assume that we have prepared in this manner a 10^{-6} *N* solution of lead nitrate, and that after evaporating it to dryness it shows a radioactivity of 10,000 relative units, then each relative radioactive unit would correspond to an amount of $2 \cdot 10^{-5}$ mg. of lead. We must of course take account of the fact that the material of the ash of the parts of the plants absorb part of the rays from the contained thorium *B*, but we can easily eliminate this disturbance by mixing the preparation used for comparison with the same quantity of ash as is contained in the sample the radioactivity of which we desire to know.

The following example shows the procedure during an experiment: *Vicia Faba* that had undergone cultivation in a nutrient solution for a fortnight was introduced, after careful washing, into 500 cc. of a 10^{-5} *N* radioactive solution of lead nitrate, which also contained 1/200 mol. of sodium nitrate. The temperature was 17°. The usual precautions such as screening the roots from light, etc., were also attended to. After 22 hours the plant was removed from the solution, and after careful washing with distilled water, the various parts—root, fruit, stem and leaves—were dried separately, ignited after the addition of a drop of concentrated sulphuric acid, and measured electroscopically[1]. The result of the experiment is shown in the following table.

Experiment (a):

Part of plant	Weight of ash in mg.	% of the total lead in the solution contained in the ash	Mg. of lead in the ash	Lead content of the ash in %
Roots	45	13·1	0·11	0·25
Fruit	5	0·10	0·0008	0·016
Stem	46	0·05	0·0004	0·001
Leaves	36	0·013	0·0001	0·0003

The purpose of the following experiments was to investigate the manner in which the assimilation of lead in the case of *Vicia Faba* varies with the lead concentration of the solution. In all of these experiments the volume of the lead solution was 200 cc. and the duration of the experiment was 24 hours.

The following collection of results shows that the individual experiments can be repeated, the agreement being quite satisfactory. Per cent. of the

[1] Before the measurement one must wait about six hours in order to be certain that radioactive equilibrium has been established between thorium *B* and thorium *C*. The reasons for this are outside the scope of this paper.

lead content taken up by the root from a 10^{-6} *N* solution: 61·2, 62·3, 57·4, 59·6, 55·4, 57·8, 47·3, 62·2, 61·7, 62·6, 60·0, 51·2, 68·7, 57·6.

Experiment (*b*). With 10^{-6} *N* lead solution:

Part of plant[1]	Weight of ash in mg.	% of the total lead in the solution contained in the ash	Mg. of lead in the ash	Lead content of the ash in %
Root	41	60·0	0·02	0·052
Stem	12·6	0·04	0·000013	0·0001
Leaves	5·5	0·004	0·000001	0·00002
Experiment (*c*). With 10^{-5} *N* lead solution:				
Root	43	31·7	0·11	0·26
Stem	18	0·015	0·0004	0·002
Leaves	9·8	0·0012	0·00003	0·0003
Experiment (*d*). With 10^{-3} *N* lead solution:				
Root	39	11·9	3·9	10
Stem	18	0·02	0·007	0·04
Leaves	18	0·002	0·0007	0·004
Experiment (*e*). With 10^{-1} *N* lead solution:				
Roots	26	0·30	9·9	38
Fruit	18	0·11	3·6	20
Stem	11	0·065	2·2	20
Leaves	10	0·035	1·2	12

[1] The fruit was removed when the plant was introduced into the culture solution, since it constitutes a particularly good nutritive medium for troublesome moulds.

From the above experimental data it is seen that, whereas in the case of a 10^{-6} *N* solution more than half of the lead is taken up by the root, the percentage loss when a 10^{-1} *N* solution is used only amounts to 0·3, although the quantities of lead taken up by the root in the latter case are very much greater than in the former case. It is of interest to note that the *percentage* of lead which passes over into the stem and leaves from the concentrated solution of lead is not smaller than that from dilute solutions. This can be interpreted as meaning that with very dilute solutions the root itself is able to bind almost the whole quantity of lead, and thus renders extremely difficult the ascent of lead into the stem and leaves. On the other hand, when a concentrated lead solution is used, an ample sufficiency of unbound lead is available, and this can be carried upwards by the transpiration current. Except in the case of concentrated solutions, the root thus protects, as it were, the remaining parts of the plant, and this marked ability for "binding" lead is probably connected with an explanation of the relatively small toxicity of lead for plants, discussed on p. 444[1] [cf. Strasburger, 1891].

On the mode of combination of lead in the root.

The question as to whether the assimilated lead enters into an organic molecule, or whether it is retained by the plant in the form of a saline com-

[1] Trees placed in solutions of copper sulphate or picric acid, etc., do not die until the poisonous substance has reached the highest points of the crown.

pound can easily be decided. In the first case, lead atoms which had once been taken up by the root would not be able to interchange places with other lead atoms, whereas in the second case an active kinetic interchange between the lead atoms bound in the plant and those present in the solution would necessarily take place.

In order to make the argument clearer, we shall designate the atoms of lead in molecules such as those of lead tetraphenyl as "red" ones, and those which occur in such a form as lead nitrate as "blue" ones. If we dissolve both compounds in the same solvent and then separate them by crystallisation, we should find only *red* atoms in the lead tetraphenyl and only *blue* ones in the lead nitrate, since the lead atoms in the lead tetraphenyl are available in an undissociable form. If, on the other hand, we dissolve equi-molecular amounts of lead chloride (with *red* lead) and lead nitrate (with *blue* lead), *i.e.* two salts in the same solvent, then after separation the two compounds would be composed half of *red* and half of *blue* lead atoms [Hevesy and Zechmeister, 1920]. The distinction between *red* and *blue* corresponds here to radioactive and to inactive lead.

If the root has taken up active lead and we place it in a solution of inactive lead, then, if the active lead lies stably embedded in organic molecules, no active lead will be able to pass over into the solution, or in other words we shall not be able to displace the active lead with the aid of inactive lead. Now experiment shows that, with the help of a solution which is relatively rich in lead (10^{-2} *N*), we can remove almost quantitatively the lead taken up by the root, whence we must conclude that *the lead in the plant root exists in the form of a dissociable saline compound*, perhaps attached to the cell walls.

For example, if we introduce a *Vicia Faba* (after careful rinsing) which has stood 24 hours in 200 cc. of an active 10^{-6} *N* lead nitrate solution into a much more concentrated 10^{-2} *N* inactive lead nitrate solution of the same volume, we find that 95 % of the active lead taken up by the root passes over into the 10^{-2} *N* solution; *i.e.* the active Pb-atoms are almost completely displaced from their places in the root by inactive atoms, which, of course, preponderate strongly (about 20,000 times), from the statistical viewpoint.

Now a 10^{-2} *N* lead nitrate solution is partially split up hydrolytically, and one might be inclined to ascribe the inverse dissolving action of lead nitrate to its acid content. However, with the aid of a 10^{-3} *N* HNO_3 solution it was possible to remove only 29 % of the lead content of the root, and by the use of distilled water as solvent only 18 % could be removed. The investigation of the assimilation of lead from solutions of different lead content showed that from 10^{-4} *N* HNO_3 64 %, and from 10^{-3} *N* HNO_3 practically the same amount, viz. 62 % is taken up by the root, when the normality of the lead ions in the solution is 10^{-6} *N*. From a 10^{-2} *N* HNO_3 solution, a concentration sufficient, in general, to kill the plant, only 26 % is assimilated by the root.

The displacement of the lead taken up by the root by other ions.

Since it has been established that we can displace the lead taken up by the root by other lead atoms, it seemed to be of interest to investigate the ability of other ions to displace the assimilated lead. 10^{-2} *N* solutions were used throughout these experiments. The plant containing lead was placed for 24 hours in the solution under consideration and then both the amount of lead remaining in the plant and the amount displaced into the solution were determined. The results of these experiments are shown in the following table:

Solution used	% of the lead initially present in the root which remained after treatment
Lead nitrate (inactive)	5
Cupric nitrate	3
Cadmium nitrate	34
Zinc nitrate	38
Chromium nitrate	43
Barium nitrate	74
Sodium nitrate	76

When the reverse solution took place with the help of a 10^{-3} *N* $Pb(NO_3)_2$ solution, 14 % of the originally assimilated lead were still present in the root after 24 hours' treatment.

Only copper is able to displace lead in a similar degree to lead itself; all the other cations investigated show an appreciably smaller displacing power.

The extent of the re-solution of the lead taken up by the stem and leaves was not determined. Experiments which are being undertaken on the assimilation of lead by *algae* will, amongst other things, also serve to shed light on this point.

As is well known, different ions are assimilated to quite different degrees by plants, according to what other ions are present in the culture solution. The toxicity of individual types of ions is also arrested by others. One of the best known cases of this "antagonism" is probably that between $CaCl_2$ and NaCl. In this case the phenomenon of the suspension of the toxicity of NaCl by $CaCl_2$ is attributed to the ability of the $CaCl_2$ to alter the plasma-membrane in such a way that it is less permeable to NaCl [Osterhout, 1912]. Since it has been possible to show that in the case of lead a kinetic displacement of the assimilated ions by other ions occurs, we shall certainly have to reckon with the possibility that the antagonism is in individual cases occasioned by such kinetic effects.

Lead assimilation and transpiration current.

From the fact that more than 50 % of the lead is taken up in 24 hours by the root in very dilute solutions of lead, *i.e.* a quantity of lead which was present in more than 100 cc. of liquid, we can conclude that it is *not the transpiration current* which transmits the assimilated lead, since the daily

loss of water of *Vicia Faba* under present conditions is less than 1 cc. This independence is also shown in the following experiment. In one case the percentage assimilation of lead by the root was determined in the usual way, and in another after the root had been first separated from the stem under water. The volume of water was 500 cc., the lead concentration was 10^{-6} N, and $p_H = 4$. Duration of experiment = 1 hour.

Root as usual	6·8 and 7·5 %.
Root cut off	6·5 and 7·1 %.

It is seen that the amount of lead taken up was in both cases the same. Moreover, the lack of dependence of the assimilation of salt on the absorption of water by the plant has repeatedly been established [cf. Arrhenius, 1922].

The toxicity of lead.

In connection with the experiments described in the previous section it is of interest to note that, as has been shown by Bonnet [1922], the introduction of plants into 10^{-1} N $Pb(NO_3)_2$ solution unfavourably influences the transpiration current. In contrast to more dilute solutions, such an appreciable concentration of lead shows distinct toxic effects on the plant[1]. *Vicia Faba* which had stood 24 hours in a 10^{-1} N $Pb(NO_3)_2$ solution already showed a slight deviation from the geotropic direction, and the leaves situated closest to the root showed signs of withering.

The toxic action of lead on different plants, such as wheat, radishes, lentils, cabbage, etc., has been investigated quite recently by Bonnet [1922]. Just as in the present case, he introduced the plants into 200 cc. of water after their roots had attained a length of several centimetres. The water contained in solution a definite amount of lead acetate or lead nitrate, and he obtained the following results:

(1) After the plants had stood in 10^{-1} N solutions of lead salts, lead could readily be detected qualitatively in the root.

(2) Only traces of lead were found in the stem and in the leaves.

(3) 10^{-1} N solutions of lead killed, *e.g.* the wheat plant after 20 days, balsam after two days.

(4) Mg, Ca and K showed no antagonistic action to lead.

(5) The greater the dilution, the less lead was taken up by the plant.

Our present results confirm those of Bonnet. As regards the first result, we were able, thanks to the sensitiveness of the radioactive method, to detect with ease and quantitatively to determine the presence of lead even in the stem and in the leaves. It is interesting to note that Mg, Ca and K, which do not have an antitoxic action, have only a slight capacity of displacing lead, according to the experiments of the present author. In reference to

[1] Cf. also Lavison [1911] and older experiments of Phillips [1883], Knop [1885], Nolle, Bässler and Will [1884].

point (5), the radioactive methods enable us to carry out a quantitative investigation of the dependence of the assimilation of lead on the concentration of the solution within wide limits, in which all other methods fail. In this manner, it is found that only 1/500 part of the amount of lead is taken up from a 10^{-6} N solution as compared with a 10^{-1} N solution. Those experiments of Bonnet should be mentioned, from which we can see the influence of the assimilation of lead on the growth of plants. He finds the following values:

Plant: The bean.

	Length of root in mm.		
	Initially	After 1 week	After 1 month
In water	25	100	1000
In 10^{-3} N $Pb(NO_3)_2$	31	31	32

SUMMARY.

(1) The assimilation of lead from lead nitrate solutions by *Vicia Faba* has been investigated. A radioactive isotope of lead was mixed with the lead nitrate, and the amount of lead taken up was determined after ignition from the radioactive intensity of the ash of the various parts. This method makes possible the determination of exceedingly small amounts of assimilated lead.

(2) Whereas 0·3 % of the lead is taken up by the root from 200 cc. of a 10^{-1} N lead nitrate solution in the course of 24 hours, 60 % of the lead content of a 10^{-6} N solution is taken up in the same time. The leaves show a lead content of only a few hundredths or thousandths of 1 % of the amount of lead present in the solution.

(3) The assimilated (radioactive) lead can be displaced by introduction of the plant containing lead into another lead solution, whereby inactive lead atoms now take the place of the radioactive ones. From this it follows that the lead is not combined with carbon within the plant, but that it exists in the form of a dissociable salt which is soluble with difficulty.

(4) Even after 24 hours, a 10^{-1} N solution of a lead salt produces toxic effects on the plant, whilst more dilute solutions do not. Lead belongs to the least poisonous of the heavy metals.

I am indebted primarily to Professor Fr. Weis for so kindly placing at my disposal the facilities of his Institute, furthermore to Dr B. Krause for his help, and to my friend Professor L. Zechmeister for numerous suggestions.

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LIV. STUDIES ON THE FAT-SOLUBLE GROWTH-PROMOTING FACTOR.

(I) STORAGE.
(II) SYNTHESIS.

By HARRY GOLDBLATT,

Beit Memorial Research Fellow;

AND

KATHARINE MARJORIE SOAMES.

From the Department of Experimental Pathology, Lister Institute.

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(I) STORAGE OF FAT-SOLUBLE *A*.

It has been known for some time that some land animals which receive the fat-soluble growth-promoting factor in their diet store a certain amount of it in their glandular organs [McCollum and Davis, 1915, 2; Osborne and Mendel, 1917, 1918; McCollum, Simmonds and Parsons, 1921; Zilva and Drummond, 1922] and a lesser amount in their body fat [Drummond, Golding, Zilva and Coward, 1920] and other tissues. Among the glandular organs the chief storehouse for this substance is the liver, but the kidneys and testes also contain a moderate amount.

The study here described is part of an investigation into the mode of action of certain forms of light in the promotion of growth and prevention and cure of experimental rickets in rats. In the course of this investigation it was found necessary to learn whether the liver of rats[1] also contains appreciable amounts of fat-soluble *A*, whether the quantity varies with that in the diet, and whether any is present in the liver of rats that have stopped growing after being fed on a diet as deficient as possible in that factor.

Experimental.

To test liver for the presence of the fat-soluble growth-promoting factor, the biological method described by Zilva and Miura [1921] was employed.

[1] Since this investigation was completed a communication has appeared on the subject of storage of fat-soluble *A* in the liver of rats [Coward, Lush and Palmer, 1923]. Their findings are very similar to those reported in Part I of this paper.

Rats were given a purified diet free from detectable amounts of fat-soluble *A*, but otherwise adequate when a sufficient amount of the above factor is present.

The diet was composed as follows:

Inactivated caseinogen	20 g.
Starch	50 g.
Cotton-seed oil (hardened)	15 g.
Salt mixture [McCollum and Davis, No. 185, 1915, 1]	5 g.
Marmite	5 g.
Decitrated lemon juice	5 cc.
Distilled water	50 cc.

When growth had ceased for about three weeks, or, after a shorter period if the animal was beginning to lose weight, the rat was considered prepared for the test and the liver feeding was begun. The liver was administered by hand to make sure that the entire amount was consumed. With few exceptions the animals ate it greedily. Liver feeding was continued for from four to six weeks, but for the sake of uniformity and of proper comparison the growth for the first four weeks only is illustrated in the charts.

The Effect of Feeding 0·5 g. of Liver from Rats receiving 3 % Cod-Liver Oil in their Diet.

Rats G. 122, G. 123, G. 126, G. 162, G. 163 and G. 165 were prepared for the test in the manner mentioned above. Of the six rats, five were actually losing weight when the special feeding was started and three had already developed varying degrees of xerophthalmia. To these six rats there was administered daily 0·5 g. of fresh liver from rats fed for from two to four months on a diet containing 5 % cod-liver oil as the main source of fat-soluble *A*. The effect can be seen in Table I and in Chart 1, in which the growth curves of rats G. 123 and G. 126 are illustrated as representative of the group[1].

During the first four weeks of liver feeding these rats gained on an average 15 g. a week, which is equal to the normal for animals of that age. The three rats (G. 122, G. 123 and G. 126) that had xerophthalmia when liver feeding was begun recovered from it in from two to four weeks. In one rat (G. 122) the cure was remarkable inasmuch as xerophthalmia was very far advanced when liver feeding was started.

In order to see whether an appreciable amount of the fat-soluble *A* contained in the liver fed to the above rats had been stored, 0·5 g. of their liver was administered to rats G. 174 and G. 175, previously prepared for the test. There was an appreciable response, but it was not nearly as great as in the case of the rats illustrated in Chart 1.

[1] In the charts only two typical representatives of all the groups are illustrated. The complete details for every group are given in Table I.

Table I. *Showing the total gain in weight of the test rats on – A diet after four weeks of additional feeding with the various amounts and kinds of liver.*

Rat No.	Type of liver tested	Amount of liver fed, g.	Total gain in weight during first four weeks of liver feeding, g.
G. 122	From rats on a diet containing 3 % cod-liver oil	0·5	59
G. 123	" " "	"	44
G. 126	" " "	"	68
G. 162	" " "	"	60
G. 165	" " "	"	71
G. 163	" " "	"	55
G. 174	From rats G. 122, G. 123, G. 126, G. 162, G. 163 and G. 165	"	22
G. 175	" " "	"	28
G. 159	From rats on a diet containing 3 % cod-liver oil	0·25	58
G. 169	" " "	"	73
G. 171	" " "	"	55
G. 170	" " "	"	45
G. 142	From rats G 159, G 169, G 170 and G. 171	"	51
G. 144	" " "	"	36
G. 482	From rats on a diet containing 10 % butter	0·5	30
G. 160	" " "	"	17
G. 177	" " "	"	20
G. 178	" " "	"	18 (2 weeks)
G. 282	From rats on a diet very deficient in fat-soluble *A*	"	Lost weight
G. 263 *a*	" " "	"	"
G. 125	" " "	"	"
G. 264 *a*	" " "	"	3
G. 278	" " "	"	0
G. 314	" " "	1·0	Lost weight
G. 500	" " "	"	"
G. 498	" " "	"	8
G. 166	" " "	"	32
G. 418	From rats on – *A* diet irradiated for four weeks after they had definitely ceased gaining weight	0·5	23
G. 523	" " "	"	36
G. 525	" " "	"	22
G. 373	" " "	"	19
G. 520	" " "	"	19
G. 375	" " "	"	13

The Effect of Feeding daily 0·25 g. of Liver from rats whose diet contained 3 % Cod-liver Oil.

After preparation, rats G. 159, G. 169, G. 170 and G. 171 were given daily 0·25 g. of liver from rats whose diet had contained 3 % cod-liver oil. Resumption of growth was prompt, and during the first four weeks the gain in weight averaged 14 g. a week, which is equal to the normal for that age. (See Chart 1 and Table I.)

The amount of fat-soluble *A* stored by rats G. 159, G. 169, G. 170 and G. 171 was now tested by feeding their liver (0·25 g.) daily, to rats G. 142 and G. 144 which had been prepared for the test in the usual way. For the effect, see Table I. Although in these two rats the growth during the first four weeks of liver feeding was greater than in those which received 0·5 g. of more potent liver, yet this is probably accounted for by the fact that rats G. 142 and G. 144 were older and heavier when they were used for the test, and such animals, according to Zilva and Miura [1921], and others, require less fat-soluble *A* to induce growth. One rat (G. 142) had mild xerophthalmia at the time liver

feeding was begun, but in ten days this had completely disappeared. When liver was discontinued, growth ceased fairly promptly, which indicates that no great amount of fat-soluble *A* contained in the 0·25 g. of this liver fed daily had been stored. (See Table I.)

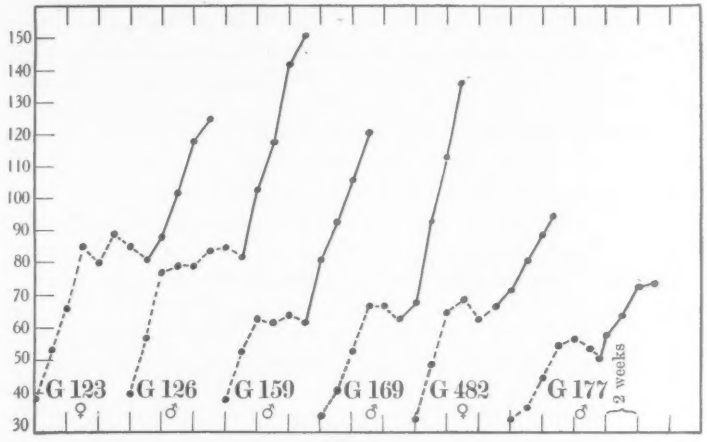

Chart 1.

G. 123 and G. 126. The effect of feeding daily 0·5 g. of the liver of rats whose main source of fat-soluble *A* was 3 % of cod-liver oil in their diet.

G. 159 and G. 169. The effect of feeding daily 0·25 g. of the liver of rats whose main source of fat-soluble *A* was 3 % of cod-liver oil in their diet.

G. 482 and G. 177. The effect of feeding daily 0·5 g. of the liver of rats whose main source of fat-soluble *A* was 10 % of butter in their diet.

Broken line – – – – = Preparatory period on $-A$ diet.

Continuous line —— = Period of liver feeding.

The Effect of Feeding Liver from Rats whose Diet contained 10 % of Butter as the chief source of Fat-soluble A.

Rats G. 160, G. 177, G. 178 and G. 482 were employed for this test and were given daily 0·5 g. of liver from rats fed during a period of from two to four months on a diet containing 10 % butter as the chief source of fat-soluble *A*. The result can be seen in Chart 1 and Table I. The difference between the growth of rats fed on the "butter liver" and that of animals fed on "cod-liver oil liver" is very striking.

Too few rats were available to permit the feeding of their liver to other rats for the detection of storage.

The Effect of Feeding 0·5 g. and 1·0 g. of Liver from rats that have ceased growing on a Diet very deficient in Fat-soluble A.

For this test many rats were placed on the diet deficient in fat-soluble *A* described at the beginning of this communication. When they had definitely ceased growing (no gain in weight for three weeks), 0·5 g. of their liver was

fed to rats G. 282, G. 264 *a*, G. 263 *a*, G. 278 and G. 125 prepared for the test in the usual way. Chart 2 and Table I show definitely that no resumption of growth occurred as a result of this addition to the diet. More rats were tried, but they are not all charted, since some lived for only a week or slightly longer after liver feeding was begun, some developed acute entero-colitis, while others refused to eat the liver after a few days and had to be discarded. None of them showed any resumption of growth; in fact the loss of weight in some was very great on account of the intercurrent intestinal infection.

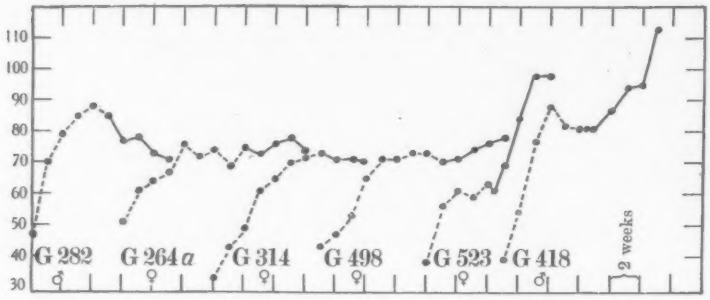

Chart 2.

G. 282 and G. 264 *a*. The effect of feeding daily 0·5 g. of the liver of rats that have ceased growing on the $-A$ diet, very deficient in fat-soluble *A*.

G. 314 and G. 498. The effect of feeding daily 1·0 g. of the liver of rats that have ceased growing on the $-A$ diet, very deficient in fat-soluble *A*.

G. 523 and G. 418. The effect of feeding daily 0·5 g. of the liver of rats on the $-A$ diet which were irradiated for four weeks after they had definitely ceased gaining weight.

Broken line - - - - = Preparatory period on $-A$ diet.

Continuous line —— = Period of liver feeding.

One gram of similar liver was fed daily to rats G. 314, G. 501, G. 498 and G. 166. Rat G. 314 died after three weeks of liver feeding, having lost slightly in weight during the period. In the case of rat G. 501 liver feeding had to be discontinued after three weeks on account of shortage of material. During the period of liver feeding it did not gain weight. This rat remained on the $-A$ diet and died one week after liver feeding was discontinued. Rat G. 498 gained 8 g. during a period of four weeks of liver feeding, while rat G. 166, receiving the same amount of similar liver, made a definite gain in weight during the first two weeks. After that growth ceased completely, the weight remained stationary, and during the last week the rat developed mild xerophthalmia. The explanation of this gain in weight during the first two weeks may lie in the fact that this rat's store of fat-soluble *A* was not completely exhausted and that the cessation of growth before the test was begun was not due to deficiency of fat-soluble *A* alone but to some complication; or, it is also possible that the liver used during that period was from rats which were on the deficient diet too short a time. However, since the deficient diet,

though very poor in fat-soluble *A* cannot be regarded as one containing no trace of that factor, it is hardly likely that the liver of animals fed on such a diet contains none of it. Therefore, it is possible that a rat stops gaining weight before the store of fat-soluble *A* in its body is completely exhausted.

Summary and Conclusions of Part I.

The liver of rats on various diets has been tested by the biological method for the storage of fat-soluble *A*.

1. The results show that the fat-soluble growth-promoting factor, if present in the diet of rats, is stored in their liver, and that it is stored in that organ in rough proportion to the amount contained in the diet.

2. In an indirect way, the conclusion of Zilva and Miura that cod-liver oil is much more potent in fat-soluble *A* than butter is confirmed.

3. Half a gram of liver from rats that had stopped growing on a $-A$ diet showed no appreciable traces of fat-soluble *A* when tested by the biological method. The effect of feeding 1 g. of such liver varied. Thus it would seem that a rat ceases growing before the store of fat-soluble *A* in its body is completely exhausted. Whether that means that this final reserve is used for some purpose other than growth cannot be deduced from these experiments.

(II) ON THE SYNTHESIS OF FAT-SOLUBLE *A* IN THE ANIMAL BODY.

It has been shown by Hume [1922] and the authors [1922] that rats on a diet very deficient in fat-soluble *A* if irradiated for about ten to twenty minutes daily by the mercury-vapour quartz lamp, grow very much better than non-irradiated rats on the same diet. Also, rats on a $-A$ diet, if irradiated daily after they have ceased gaining weight, resume growth for a variable period. But in both cases gain in weight eventually ceases and the subsequent history is the same as that of non-irradiated rats, viz. decline in weight, development of xerophthalmia in some of the animals, and finally death with or without a complicating terminal infection. Because irradiation was unable to effect the continuance of growth on a $-A$ diet, the assumption of a photosynthesis of fat-soluble *A* in the animal body to account for the stimulation of growth seemed unjustified. But it was suggested that the mode of action of light in stimulating growth was "by effecting an economy of the action of the fat-soluble factor still stored in the animal at the time irradiation was begun, as well as by making available the small quantities of organic factor which may still be present in the daily ration of deficient diet."

At the same time that the above investigation was being carried out another method was used to determine whether synthesis of fat-soluble *A* occurs in the body of a rat under the influence of rays from a mercury-vapour quartz lamp.

It has been shown in the first part of this communication that 0·5 g. of the liver of rats on a $-A$ diet that have ceased gaining weight for about three weeks contains no fat-soluble *A* detectable by the biological method, and that

1 g. of such liver gave a positive result in only one out of four tests. Rats were therefore placed on the $-A$ diet, and when they had ceased gaining weight for three weeks, at which time but little fat-soluble A should have been left in the liver, irradiations by the mercury-vapour quartz lamp were begun. In most instances (as reported before) resumption of growth occurred promptly and lasted for a variable period, but rarely for longer than four weeks. Therefore, after four weeks of irradiation, when they had again stopped gaining weight, the rats were killed and their liver was tested in the usual way for fat-soluble A. In the case of rats G. 418, G. 523 and G. 525 the gain was as great or greater than that caused by the same amount of "butter liver" (see Chart 2). Rats G. 373, G. 520 and G. 375 did not show as great a gain (see Table I), but the liver feeding was carried out for only two weeks on account of shortage of material. In all three cases the rats ceased gaining weight quite promptly and died in from three to six weeks after the liver was discontinued. Rats G. 523 and G. 525 were kept on the $-A$ diet for about two weeks after liver feeding was discontinued, during which time they lost weight; and then 0·25 g. of liver from rats receiving cod-liver oil was administered to them. In both cases there was a very prompt gain in weight.

Discussion.

The interpretation of the above results is difficult. For reasons already stated it is not justifiable to postulate actual synthesis of fat-soluble A in the body of these rats[1]. If not from synthesis, then where did the vitamin in the liver of the irradiated animals come from? One possibility, which has not yet been tested experimentally, is that it is mobilised from the other parts of the body in which it occurs and is partly stored in the liver. Then why, despite the appreciable store of fat-soluble A still present in their liver, did the irradiated rats not continue to grow, while their liver, when fed to test animals, stimulated the growth of these? Apparently, in the irradiated rats, the vitamin, though still present in the liver cannot be liberated and so be made available to the organism, while that same liver when ingested by another rat becomes a direct source of supply of fat-soluble A immediately available by absorption from the intestinal tract. It must be remembered that during the experimental period of four weeks a test rat consumed about four livers from irradiated animals. But without definite knowledge of how and where in the body this vitamin acts one cannot state why the ingested liver should have this effect. The correct explanation must await the results of further studies on the nature of the fat-soluble growth-promoting factor as well as on the site and mode of its action.

[1] The photosynthetic production, however, of fat-soluble A from some stored material which is not renewed on the deficient diet, is not absolutely excluded.

Summary and Conclusions of Part II.

1. Half a gram of liver from rats on a $-A$ diet that have ceased gaining weight for at least three weeks contains no fat-soluble A detectable by the biological method.

2. Half a gram of liver from rats on the same $-A$ diet irradiated by the mercury-vapour quartz lamp for four weeks after they have definitely ceased gaining weight does contain an appreciable amount of fat-soluble A detectable by the biological method.

3. The results of the above investigation are not interpreted as evidence of synthesis of fat-soluble A in the animal body.

(Note added on 23rd August, 1923.)

In a preliminary investigation by one of the authors (S.) on the storage of the *antirachitic* organic factor, definite protection against the development of rickets in rats on diet 3143 of McCollum, Simmonds, Shipley and Park [1921] was afforded by the daily administration of 1 g. of liver from rats on purified casein diet which were given 100 milligrams of crude cod-liver oil every day. Partial protection was obtained by 0·5 g. of liver.

But 1 g. of liver from rats, that had ceased growing on the purified diet alone, did not afford any protection against the development of rickets on diet 3143.

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LV. THE TYROSINASE-TYROSINE REACTION.

By HENRY STANLEY RAPER and ARTHUR WORMALL.

From the Department of Physiology and Biochemistry, the University of Leeds.

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Tyrosinase, an oxidising enzyme which is widely distributed in the vegetable kingdom and has also been met with in animal organisms, has formed the subject of many investigations since it was discovered by Bourquelot and Bertrand [1896]. The enzyme differs from other oxidation catalysts in that it is able to bring about the oxidation of tyrosine by atmospheric oxygen. Laccase, for instance, which is usually considered to contain an oxygenase-peroxidase system, and hydrogen peroxide and a peroxidase, are without effect on tyrosine solutions. The action of the enzyme on tyrosine is accompanied by the development of a red colour, which gradually becomes reddish brown and finally a black pigment (melanin) is deposited. The nature of the action has been a matter of dispute and has been attributed to a hydrolytic deaminase, a phenolase or oxygenase-peroxidase system, to an enzyme which splits the tyrosine by hydrolysis, and to the inorganic salts associated with the enzyme acting as condensing agents on the oxidation or hydrolytic products produced by the enzyme itself. Into the merits or demerits of these various theories we do not wish to enter at present. Our interest was directed to one important factor concerning the action of the enzyme by the observation that the reaction of the medium in which the enzyme acts has a very marked influence on the colour development both as regards the rate of its production and the tint obtained, and also, that if a partially oxidised tyrosine solution is heated, the red substance first formed is converted into one which is colourless. This colourless substance, while being stable for many hours in an acid solution, rapidly gives rise to a bluish-black pigment on being made alkaline with sodium carbonate. From these simple observations, it was inferred that any investigation of the action of tyrosinase must be carried out with careful regard to the p_H of the medium. This is especially true when work bearing on the activity as co-enzymes or inhibitors of various substances or extracts is concerned, and also when the action of tyrosinase on various substances other than tyrosine is being investigated. Our observations have led us to conclude that because of the neglect to work with buffered solutions, a large number of erroneous conclusions have been drawn regarding the enzyme and its action.

The work described in the present paper has defined the limits of p_H at which the enzyme is active, and the velocity of the action at three different values of p_H has been measured. It has also been shown that the accelerating effect of boiled potato juice on the enzyme action which has been ascribed by Haehn [1919] to the salts present, is not due to the salts but to some organic constituent of the juice, since the ash of the boiled juice does not show the same effect. The results arrived at by Haehn are undoubtedly due to working with unbuffered solutions, a conclusion arrived at independently by Chodat and Wyss [1922].

From what has been mentioned above, it is apparent that previous measurements of the velocity, in which the p_H was not kept constant or in which the measurements of the rate of oxidation have been carried out by colorimetric methods, are unreliable. The studies by von Fürth and Jerusalem [1907] and by Bach [1908] come into this category. Bach did not use a colour comparison for estimating the degree of oxidation but employed an empirical procedure by oxidising with permanganate in acid solution the pigment formed during the action of the enzyme. Whilst it is true that the pigment formation from tyrosine is a notable feature of the enzyme action, it is certainly true that more than one pigment is formed, and it is also possible that non-pigmented substances may first be formed from the tyrosine [Bach, 1914]. The permanganate method therefore appears to us to be too crude. The method used in the present work was to control the p_H rigidly by using well-buffered solutions, and to estimate the tyrosine at the various stages of the reaction by bromination. In this way, satisfactory curves have been obtained which indicate that as regards the disappearance of the tyrosine, the reaction is of the unimolecular type and that the enzyme is therefore a true catalyst. This observation is of special interest if an oxygenase is a constituent of the enzyme. The oxygenases are supposed to be autoxidisable substances which on exposure to air form peroxides. In the presence of a peroxidase these peroxides are decomposed and yield "active" oxygen. Substances such as aldehydes or unsaturated fatty acids are usually referred to as bodies which are similar to the oxygenases since they are known to form peroxides on exposure to air. They would not function as true catalysts, however, because the peroxides which they form, do not yield the original aldehyde or unsaturated acid on decomposition, but rather, oxidation products of these substances. Thus benzaldehyde gives rise to benzoic acid and active oxygen.

$$C_6H_5.CHO + O_2 \longrightarrow C_6H_5.\overset{\overset{O}{\|}}{C}—O—OH$$

Benzoyl hydrogen peroxide

$$C_6H_5.\overset{\overset{O}{\|}}{C}—O—OH \longrightarrow C_6H_5.COOH + O \text{ (active)}$$

Benzoic acid

If the oxygenases were of this type, their concentration in the enzyme system would gradually diminish as the reaction proceeded and the velocity

constant would diminish considerably. This we have not found to be the case with tyrosinase and it indicates that if an oxygenase is a component of the enzyme, it acts as a true catalyst and is not permanently altered in bringing about the transfer of atmospheric oxygen either to the tyrosine or to its decomposition products produced by some other component of the enzyme.

Some of the observations recorded in the present paper point clearly to the existence of definite stages in the colour changes which occur when tyrosine is oxidised by the enzyme. These stages appear to be due to the formation of definite compounds which are intermediate between tyrosine and the final product, melanin. They are produced under definite conditions which have now been ascertained and it is hoped eventually to succeed in their isolation and identification. If the enzyme is allowed to act at p_H 6, the initial product is bright red in colour and with this reaction of the medium and in the cold, it is fairly stable, changing only slowly into the black pigment which is the end product of the reaction. If the oxidation is carried out at p_H 8·0, the red substance does not accumulate, but is rapidly transformed into the black melanin. The red substance, therefore, is to be regarded as the initial product of the reaction. It is not transformed directly into melanin, however, but rather into a colourless substance, which then undergoes oxidation to give melanin. This takes place rapidly in alkaline solutions. The evidence for this is that solutions of the red substance become colourless if allowed to stand in an oxygen-free atmosphere. The change takes place slowly in the cold, more rapidly on warming, and is almost instantaneous when the solution is boiled. If the colourless substance thus obtained be made alkaline, it rapidly darkens in the air, and finally deposits a black precipitate. Bach [1914] has also observed that the red substance is decolorised on heating or on standing in an inert atmosphere, but his conclusions about the change are very different from those we have reached. He attributes the decolorisation of the red substance to a process of reduction and suggests that the reducing agent concerned is a hydrolytic aminoacidase which he regards as a component of the enzyme. Although there appears to be satisfactory evidence that tyrosinase will deaminise amino-acids, the observations of Bach are the only ones which suggest that the action is a hydrolytic oxidation. The evidence submitted by him is, however, unconvincing, and in the present paper experiments are described which show that Bach's conclusions regarding the nature of the enzyme are erroneous. We have been unable to obtain any evidence that a hydrolytic oxidation is taking place. The red substance, it is true, becomes colourless on reduction by SO_2 as well as on simple standing in an inert atmosphere. It could therefore act as a hydrogen acceptor and be reduced if a hydrolytic oxidation catalyst were present. The only evidence submitted by Bach which is in favour of a reduction process is that the decolorisation of the red substance takes place in an inert atmosphere and that if the solution be then shaken in air, the colourless substance is reoxidised and becomes red. To show this, Bach takes a solution of tyrosine, adds tyrosinase and shakes

well for a few minutes at 35–40°. The tyrosine is oxidised and becomes red. The solution is then placed in an atmosphere of nitrogen and becomes colourless (according to Bach, by reduction). On readmitting air and shaking, it again becomes red. According to Bach, this is due to reoxidation of the colourless substance. It may, however, equally well be due to the further action of tyrosinase on tyrosine (both being present), which reaction can now proceed, oxygen having been admitted. That the latter is the true explanation is shown in the present paper. Solutions containing the red substance free from tyrosinase have been prepared. They become colourless on standing in an inert atmosphere, and do not give the red substance again on exposure to air; instead, melanin is produced; slowly if the solution be acid, quickly if it be alkaline. In this experiment, no enzyme is present when the red substance changes to the colourless compound, and the change, therefore, cannot be due to an enzymic reduction. Furthermore, tyrosinase in the presence of tyrosine will not reduce methylene blue in an inert atmosphere. If a hydrolytic oxidation catalyst were present, then the methylene blue ought to act as a hydrogen acceptor and be reduced. The most probable explanation of the above-mentioned colour changes is that the red substance is converted into the colourless compound by intramolecular change, this being slow in the cold, but rapid on heating.

In the light of the evidence presented in this paper, therefore, the action of tyrosinase on tyrosine is divisible into three stages.

1. The conversion of tyrosine into a red substance. This requires the presence of oxygen and the enzyme.

2. The conversion of the red substance into a colourless substance. This is spontaneous, and takes place rapidly on warming. It does not require the presence of tyrosinase and is probably an intramolecular change which the red substance undergoes.

3. The oxidation of the colourless substance by oxygen to melanin. This may take place spontaneously but may also be accelerated by the phenolase which is present in tyrosinase. It is rapid in alkaline solutions, but slow in acid solution.

Experimental Part.

The source of tyrosinase and its partial purification.

In certain reactions, it is essential that the tyrosinase should be free from some of the substances usually associated with the enzyme, and different methods of purification have been used according to the nature of the substances which were to be removed. In many cases, however, ordinary potato juice was found to give results identical with those obtained when the purified enzyme was used, and in these cases, untreated potato juice was used. This juice was obtained by washing ordinary market potatoes, mincing and then pressing in a meat press. The juice was allowed to stand for a short time and then centrifuged to remove starch.

The purified enzyme solutions most used in these experiments have been obtained by the dialysis of ordinary potato juice. The latter was contained in a very permeable collodion sac suspended in flowing water, and dialysed for four or five days under a slight pressure, this preventing undue dilution of the enzyme solution. The solution obtained in this manner does not give a reaction with tyrosine as good as that given by the original potato juice, but its activity can be increased by vigorous shaking after addition of sufficient sodium carbonate to make the solution 0·1 %.

Another method of purification depended on the digestion of the proteins of potato juice by pancreatic extracts. After four days' digestion in an incubator, the solution was acidified with dilute acetic acid until a definite and approximately maximum precipitate was obtained, care being taken to avoid excess of acetic acid. The precipitate was filtered off, washed with water, and suspended in 0·1 % sodium carbonate solution, in which it dispersed fairly well. It gave a good reaction with tyrosine, though only about half as intense as that given by a corresponding volume of the original potato juice.

Use has also been made of a fairly pure enzyme obtained by repeated precipitation with alcohol to 40 %, and suspension of the precipitate in 0·05 % or 0·1 % sodium carbonate solution. This method probably yields an enzyme preparation similar to that obtained by the method described by Chodat [1912].

The influence of the hydrion concentration on the action of the enzyme.

It has been found that the hydrogen ion concentration of the medium has a marked influence not only on the velocity of the enzyme action, but also on the nature of the pigments produced from tyrosine.

Buffered solutions containing 0·05 % of tyrosine were obtained from phthalate solutions, phosphate solutions, or boric acid and NaOH mixtures, according to the tables given in Cole's practical *Physiological Chemistry* and Clarke's *Determination of Hydrogen Ions*. 50 cc. of each solution were measured into a 200 cc. Erlenmeyer flask, 5 cc. of fresh potato juice added, and allowed to stand at the room temperature (15°). The solutions were shaken every half-hour, and at intervals were poured out into boiling tubes, any colour changes being noted.

p_H 4·0. No apparent oxidation, *i.e.* no colour changes.

p_H 5·0. Very little oxidation. Slight red colour after 5 or 6 hours.

p_H 6·0. Rapid oxidation. A deep red colour after one hour becoming very intense after 3 or 4 hours. At the end of 24 hours, the solution had become much darker, though the red phase was still very evident.

p_H 7·0. Rapid oxidation. A deep red colour after 1 hour, slightly blacker than that at p_H 6·0. The solution remained blacker than that at p_H 6·0 throughout the experiment and melanin formation was more rapid.

p_H 8·0. Rapid oxidation. After one hour, the solution had a red colour, which gradually became darker and more intense. The subsequent blackening and the formation of melanin were more rapid than in the solution at p_H 7·0.

p_H 9·0. Slight blackening with a little red colour after 2 hours. After 12 hours the solution was very dark, though much lighter than that at p_H 8·0.

p_H 9·5. Similar results to those at p_H 9·0, with corresponding colours of less intensity. Thus there is less visible oxidation than at p_H 9·0.

p_H 10·0. Very little oxidation. Slight darkening after 6 hours.

p_H 10·5, 11·0, 12·0. No apparent oxidation.

The solutions were also tested at intervals for the relative amounts of red substance present in each. This was carried out by withdrawing 5 cc. from each solution, adding one drop of 10 % acetic acid, boiling, filtering and adding sodium carbonate to the filtrate until it was definitely alkaline. By this treatment, the red chromogen is converted into the colourless substance by the process of boiling, which also destroys and coagulates the enzyme, while the addition of the alkali causes the rapid conversion of the colourless substance into melanin. This last reaction yields a bluish-black solution, and the degree of intensity can be regarded as an approximate measure of the amount of red substance present in the original sample.

The results show that the formation of this red chromogen is at a maximum at p_H 6·0 to 6·5, is less at p_H 7·0, and that the amount then decreases rapidly with increasing alkalinity. As the enzyme action takes place at p_H 8·0 with a velocity equal to, or slightly greater than, that at p_H 6·0, the small amount of red substance found at p_H 8·0 must be due to the rapid conversion of this substance into melanin.

The limits of oxidation of tyrosine by tyrosinase under the conditions given above, are p_H 5·0 and p_H 10·0 and the enzyme seems to be most active in neutral, slightly alkaline or slightly acid solutions, *i.e.* in the range p_H 6·0 to 8·0. Even over this range, however, the colour changes vary enormously.

Chodat and Wyss [1922] working with unbuffered solutions have obtained similar limits of p_H for the tyrosinase-tyrosine reaction, though their limit for the alkaline solutions (p_H 11·0) is somewhat higher than that observed in the experiments described above.

It is of interest that Mrs E. C. Venn [1920] working with a cheese organism which produced colour changes in tyrosine solutions found that the limits of p_H within which the colour was obtained were 3·23 and 9·7. The closeness of these limits to those observed by us suggests that the organism in question produced tyrosinase. The non-correspondence in the more acid media is possibly to be explained by the fact that the enzyme is precipitated in solutions at p_H 5·0, whereas it might still be active inside the bacteria.

The nature of the changes occurring in the tyrosinase-tyrosine reaction.

The first process in the oxidation of tyrosine by tyrosinase is the formation of a red pigment and this only takes place in the presence of tyrosinase and oxygen. The formation of this red substance can best be shown by the partial oxidation of a saturated tyrosine solution at p_H 6·0 by means of one-tenth its volume of fresh potato juice. This product is relatively stable in acid solutions, but in an alkaline medium it is rapidly converted into melanin, probably with intermediate formation of a colourless substance.

If a partially oxidised tyrosine solution at p_H 6·0 is treated with colloidal ferric hydroxide to remove the enzyme and other colloidal substances, a clear deep red solution is obtained which is fairly stable, but on standing several hours it deposits a black pigment, melanin. This blackening, which is a process of oxidation, continues until all the red colour disappears. At higher temperatures such as 35–40°, the decolorisation of the red substance is more rapid, but no blackening takes place unless the solution has free access to oxygen. If the red solution is boiled, immediate decolorisation takes place and subsequent exposure to the air causes the precipitation of melanin. In this way, by the use of colloidal ferric hydroxide to remove tyrosinase from the coloured solutions, Bach's theory [1914, p. 222] that tyrosinase contains a hydrolytic oxidation enzyme and that the conversion of the red substance into the colourless compound is an enzymic reduction process is disproved. It has also been found that the decolorisation of the red substance takes place spontaneously, though it may be accelerated by the enzyme, possibly by a process of adsorption. It takes place in the absence of oxygen, and can be quickened by raising the temperature, taking place extremely rapidly at 100°. These, and certain other facts suggest to us that the process is due to an intramolecular change.

In repeating the experiment of Bach [1914, p. 222) the red solution obtained by the action of 1 vol. of potato juice on 10 vols. of a saturated tyrosine solution at p_H 6·0, which contained tyrosine, tyrosinase, the red substance, and possibly some of the colourless compound, was divided into two parts. One half was treated with colloidal ferric hydroxide and filtered. A clear red solution was obtained which contained no enzymes. This filtrate and the other half of the original red solution containing tyrosinase were placed in flasks, the latter evacuated to remove all the oxygen, and then filled with CO_2. They were then made airtight with rubber stoppers and placed in an incubator at 37°. After some time both solutions became colourless, but on shaking with air, only that containing tyrosinase became red again. Thus the subsequent reddening obtained by Bach, and attributed by him to the reoxidation of the colourless substance, is really due to the renewal of the action of tyrosinase on tyrosine, both of which are still present in the solution. This experiment shows clearly that the decolorisation of the red substance is not a process of reduction by the enzyme, for it takes place in the absence of the latter, and

also the colourless substance cannot be oxidised to the red compound again by shaking with air or oxygen.

Other evidence pointing to the non-existence of a hydrolytic oxidation enzyme in potato juice is our inability to obtain any oxidation of tyrosine by tyrosinase in the absence of oxygen and in the presence of methylene blue as a hydrogen acceptor. Buffered 0·05 % tyrosine solutions at p_H 6·0 and 8·0 with one-tenth their volume of potato juice or dialysed potato juice do not undergo any visible oxidation in an atmosphere of hydrogen or *in vacuo*, even if varying amounts of methylene blue are previously added. No reddening or darkening of the solutions occurs, even after 48 hours in an incubator, but subsequent admission of air causes fairly rapid oxidation, showing that the enzyme has not lost its activity towards tyrosine.

The kinetics of the tyrosinase-tyrosine reaction.

In the following experiments, an attempt has been made to study this oxidation process on quantitative lines, to determine the "order" or type of the reaction, and also to make a careful study of the effects produced by adding to the enzyme certain constituents of potato juice which by themselves have no action on tyrosine. The results obtained indicate that the reaction is of the ordinary uni-molecular type and that the activity of the enzyme decreases very little during the reaction. Further, it is shown that in order to make any useful comparison, it is essential that the solutions used should be at the same hydrogen ion concentration, and should have an efficient buffering capacity. Thus we can confirm the statement of Chodat and Wyss [1922], that the results previously obtained by Haehn [1920] were simply due to alterations in the hydrogen ion concentration of the solution. In one respect, however, we have confirmed Haehn's results and find that potato juice rendered almost inactive by dialysis can be activated by the addition of certain mineral salts. This activation is quite different from that brought about by boiled potato juice, and our experiments show that the latter effect is not due to inorganic salts. It is found that certain samples of boiled potato juice, which are themselves inactive towards tyrosine, are able to activate the tyrosinase of potato juice, but the nature of this "co-enzyme" or activator is uncertain. This "co-enzyme" is relatively heat stable, but is not in the nature of an inorganic salt, because the ash of the same boiled potato juice has no activating power.

In order to follow quantitatively the relations existing between the enzyme and the substrate, only one of the two usual procedures has been used. Tyrosine being only slightly soluble in water, it is not possible to allow varying amounts of the enzyme to act upon a tyrosine solution and to assume that the concentration of the substrate remains constant during the experiment. In consequence, the rate of disappearance of tyrosine in the presence of a constant amount of enzyme has been followed. The method is laborious and time consuming, since the intermediate products of the oxidation as well as

the final product, melanin, have to be removed before the tyrosine can be estimated. A colorimetric estimation involving the use of a phosphotungstic and phosphomolybdic acid reagent, was found to have no advantage in this respect over the older method of bromination, so the latter was adopted. Errors due to bromine-absorbing compounds other than tyrosine, which were present in the enzyme solution, were eliminated by the use of controls.

General method of procedure. The method consisted of placing the tyrosine and control solutions contained in 500 cc. round-bottomed flasks in a thermostat at 20° and drawing through each solution a constant current of air saturated with water vapour and toluene. All the solutions were buffered by means of mixtures of NaOH and KH_2PO_4 solutions, and, by the addition of distilled water when necessary, it was arranged that all the solutions in comparative experiments should have the same total volume. In the case of the tyrosine solutions, 300 cc. of a phosphate buffered 0·05 % solution of tyrosine were used in each experiment, and for each of these solutions a control was set up, using the same quantity of distilled water and buffering solutions. Equal amounts of potato juice were added to all the solutions, together with 10 cc. of toluene, the latter preventing frothing and bacterial decomposition. The potato juice used was obtained by the process described above (p. 457). This juice was not purified in any way since the bromine absorbing capacity did not prove to be very high.

Samples (20 cc.) were withdrawn from each solution at the commencement of the reaction, and also after certain intervals. Each sample was immediately treated with 0·5 cc. of 10 % acetic acid and boiled to destroy the enzyme and coagulate the protein matter. After being plugged with cotton-wool, the flask containing the sample was allowed to stand for two days. The solution was then filtered, the precipitate of melanin and protein coagulum being washed several times with hot water to remove all the tyrosine adhering to the precipitate, and the filtrate made alkaline by the addition of 1 cc. of 10 % sodium carbonate. The solution was then boiled, the flask plugged with cotton-wool, and allowed to stand 24 hours. 1 cc. of 10 % acetic acid was then added, the solution boiled, allowed to stand for a few hours, and finally filtered. The tyrosine in each sample was then estimated by the method described below. This treatment of the samples was the one found most satisfactory in removing the intermediate products of oxidation together with most of the substances present in potato juice which absorb bromine. In spite of this very drastic treatment, however, the samples from the controls, *i.e.* the solutions containing only the buffering salts and potato juice, absorbed a small amount of bromine, but this remained almost constant throughout the experiment. Thus, by subtracting the "tyrosine figure" of the control from that of the corresponding tyrosine solution, a true "tyrosine figure" has been obtained for each sample and these enabled us to calculate the concentration of tyrosine in each solution after the various intervals of time.

These figures of tyrosine content have been used directly to draw curves

showing the relationship between the concentration of tyrosine and the time of reaction, and have also been used to calculate the values of k, the velocity coefficient, on the assumption that the reaction is of the uni-molecular type. The constancy of k throughout the experiments indicates that this assumption is correct, and thus the velocity of the reaction at any particular time is directly proportional to the concentration of tyrosine at that moment.

The estimation of tyrosine. The method, which is a modification of that used by Millar [1903], consists of adding to each tyrosine solution a standard solution of bromine containing more bromine than can be absorbed by the tyrosine and other bromine-absorbing substances present and estimating the excess.

The tyrosine solution was treated with 10 cc. of standard $NaBrO_3$ (0·8502 g. per litre; 1 cc. = 0·001527 g. tyrosine) solution, 2 cc. of 50 % KBr solution, and 7·5 cc. of 20 % HCl solution, the flask corked and allowed to stand 20 minutes. 2 cc. of 10 % KI solution were then added, and the free iodine titrated with $N/50$ $Na_2S_2O_3$ solution. After making allowance for the bromine absorption of the control, the true tyrosine content was calculated as below, but in some cases the differences between the sodium thiosulphate titration figures of each sample and the corresponding control were used to represent the relative concentrations of tyrosine.

The $Na_2S_2O_3$ solution was first standardised by liberating a known amount of bromine from solutions of $NaBrO_3$, KBr and HCl, replacing the free bromine by iodine, and titrating the latter with the thiosulphate solution.

All the following experiments were carried out in a thermostat regulated at 20°. A qualitative experiment showed that the rate of oxidation increased with rise of temperature.

Comparison of the influence of air and oxygen on the rate of oxidation. In all the experiments, a constant current of air was drawn through each solution during the whole of the reaction under observation, and thus it was first necessary to prove that the oxygen supply was adequate. If this were not so, the reaction followed would merely be the enzyme action controlled by a limited oxygen supply, and if the enzyme is of the ordinary oxidase type, that reaction would be the rate of formation of the peroxide.

The method adopted to prove this point before passing on to the more important investigations, was to compare the rate of oxidation under these conditions with the rate when the oxygen supply was increased five-fold by replacing the air current by an oxygen current approximately equal in volume.

Solution *A*. 300 cc. phosphate buffered 0·05 % tyrosine solution (p_H 8·0) + 30 cc. potato juice + 10 cc. toluene.
" *B*. 300 cc. phosphate buffered 0·05 % tyrosine solution (p_H 8·0) + 30 cc. potato juice + 10 cc. toluene.
Control *A*. 300 cc. phosphate buffered water (p_H 8·0) + 30 cc. potato juice + 10 cc. toluene.
" *B*. 300 cc. " " " "
Air was passed through *A* and Control *A*. *Oxygen* through *B* and Control *B*.

Samples (20 cc.) of each of the four solutions were then taken at the commencement of the reaction, and after certain intervals, and after being treated

as indicated previously, the tyrosine in each was estimated by the bromination method.

Results. Amount of tyrosine (g.) present in the original volume of solution (330 cc.):

A (air)	0·1437	0·1275	0·0954	0·0716	0·0573
B (oxygen)	0·1437	0·1272	0·0935	0·0748	0·0574
Time (mins.)	0	67	160	250	315

The control figures remained constant, or the variations were within the limits of experimental error, and both controls gave the same titration figures.

The above results prove conclusively that the oxygen supply is adequate if a current of air similar to that used in this experiment is drawn through each solution and if this supply is maintained throughout the experiment. It may also be noted that this current of air also keeps the solution well agitated and so prevents differences in temperature or local concentration of enzyme.

Influence of hydrion concentration on the velocity of oxidation. In this experiment, three tyrosine solutions (*D*, *C* and *B*) were prepared, buffered by phosphate solutions at p_H 6·0, p_H 7·0 and p_H 8·0 respectively, and a control (*A*) was set up, using the same amount of phosphate solution at p_H 6·0. A preliminary experiment proved that controls at the three different hydrion concentrations gave equal thiosulphate titration figures, and so only one control was necessary for this comparative experiment. To 300 cc. of each of the solutions *B*, *C*, and *D* and solution *A* 30 cc. of potato juice were added and 10 cc. of toluene, and they were then placed in the thermostat at 20°. The same current of air was passed through all four solutions. Duplicate samples were withdrawn at intervals and treated as described above.

Results. Grams of tyrosine in 330 cc.:

B. p_H 8·0	0·1198	0·0911	0·0781	0·0706	0·0629	0·0561	0·0148
C. p_H 7·0	0·1179	0·0973	0·0821	0·0772	0·0688	0·0603	0·0214
D. p_H 6·0	0·1168	0·0963	0·0855	0·0796	0·0730	0·0622	0·0339
Time (mins.)	0	135	233	313	418	485	1420

Velocity coefficients. These were calculated on the assumption that the reaction is uni-molecular, *i.e.* $k = \frac{1}{t} \log_e \left(\frac{a}{a-x}\right)$ or $k = \frac{2{\cdot}302}{t} \log_{10} \left(\frac{a}{a-x}\right)$, where a = the original concentration of tyrosine and $(a - x)$ the concentration after time t.

Velocity coefficients (k).

							Average
B. p_H 8·0	0·0020	0·0018	0·0017	0·00155	0·0016	0·0015	0·0017
C. p_H 7·0	0·0014	0·0015	0·0014	0·0013	0·0014	0·0012	0·0014
D. p_H 6·0	0·0014	0·0013	0·0012	0·0011	0·0013	0·0009	0·0012
Time (mins.)	135	233	313	418	485	1420	—

This experiment was repeated later with another enzyme preparation, and the following figures obtained:

Velocity coefficients (k).

					Average
p_H 8·0	0·0023	0·0023	0·0022	0·0019	0·0022
p_H 7·0	0·0015	0·0017	0·0020	0·0019	0·0018
p_H 6·0	0·0015	0·0015	0·0015	0·0013	0·0015
Time (mins.)	66	188	375	1440	—

The curves obtained by plotting the concentration of tyrosine against the time are of the true logarithmic type, indicating that the amount of tyrosine oxidised during any short interval of time is directly proportional to the average concentration of tyrosine during that interval. In Fig. 1 the curve at p_H 8·0 was obtained by substituting for k in the formula $k = \frac{1}{t} \log_e \frac{a}{a-x}$, the average value for k found in the experiment at p_H 8·0, and calculating from this the corresponding values of $(a - x)$ or x for different intervals of time. Points are also marked on the graph which represent the concentrations of tyrosine actually found by estimation, and it will be seen that these are in close agreement with the average curve. Similar curves are also drawn giving the oxidations at p_H 6·0 and p_H 7·0, and it will be observed that the velocity is greatest at p_H 8·0 and least at p_H 6·0.

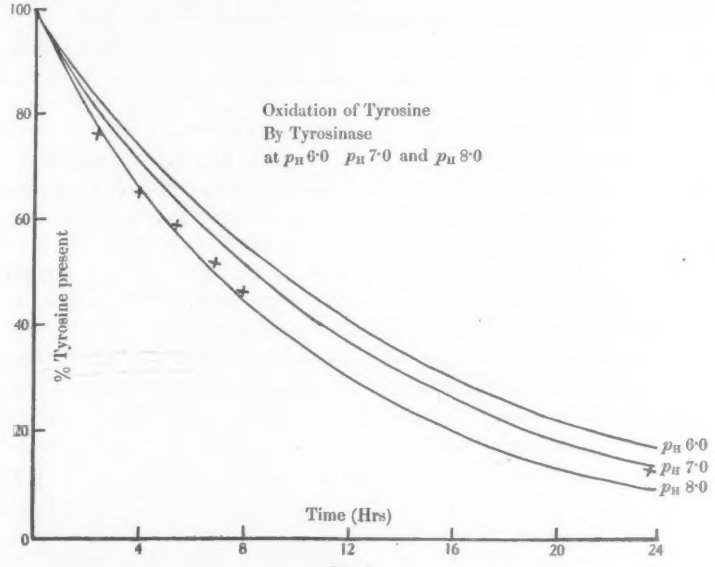

Fig. 1.
× — Experimental points for p_H 8·0.

The approximate constancy of the values of the velocity coefficient during the whole of the reaction proves that the reaction is of the uni-molecular type and is therefore comparable with other enzyme actions. In this constancy of the values of k, however, the reaction seems to deviate somewhat from those followed by other workers. Thus in the case of the hydrolysis of milk-sugar by lactase [Armstrong, 1904] the value of the velocity coefficient falls rapidly from 0·0640 for the first hour to 0·0310 for 5 hours and 0·0129 for 24 hours. In the case of the hydrolysis of cane-sugar by invertase [Henri, 1903] the velocity coefficient steadily increases from 0·00058 during the first

hour to 0·00085 during 11 hours. This constancy in the case of the tyrosinase-tyrosine reaction is probably due to the absence or elimination of certain factors which would have an accelerating or retarding effect on the reaction. Thus the nature of the reaction seems to preclude the possibility of a reverse reaction playing any part in the process, while effects due to changes in hydrion concentration may be ruled out on account of the buffering of the solution. With regard to the destruction or change in the nature of the enzyme, our observations lead to the conclusion that tyrosinase is not very unstable at these hydrion concentrations.

The values obtained for the velocity coefficient fluctuate during the experiment or show a slight decrease during the oxidation of half the tyrosine, followed by a larger decrease during the oxidation of the remainder. These divergences during the first six or eight hours are not very great, except perhaps at p_H 8·0 and in view of the small amount of tyrosine present in each sample (0·0075 g. in the first samples and about 0·0010 g. in the last) and the rigorous treatment to which these samples must be submitted, these divergences are not greater than the experimental error involved in their determination. With reference to the "slowing" which occurs during the second part of the reaction, it is interesting to note that this is less marked in the experiments carried out at p_H 7·0 and this indicates certain factors which may be the cause of retardation. In acid solutions, the enzyme is precipitated, and although this is not very marked at p_H 6·0, it will have an appreciable effect on reactions proceeding for 24 hours, and will cause a decrease in velocity similar to that observed. With regard to the decrease at p_H 8·0, no destruction or precipitation of the enzyme should take place, because tyrosinase seems to be very stable and well dispersed at this hydrion concentration, and the determining factor appears likely to be the adsorption of the catalyst by the melanin produced, this being the main product of oxidation under these conditions.

The results obtained indicate that slight alkalinity favours the action of the enzyme, for the velocity at p_H 8·0 is greater than that at p_H 7·0, and this in turn is greater than that at p_H 6·0. The exact conditions determining this greater velocity at p_H 8·0 are not known, but it is suggested that there are two factors which probably play a very important part in this phenomenon. Firstly, the dispersion of the enzyme, as judged by the naked eye, is best at this hydrion concentration, and secondly, if the first reaction, the conversion of tyrosine into the red pigment, tends to be at all reversible, this will have least effect at p_H 8·0, because of the rapid conversion of the intermediate products into the final product, melanin.

The effect of adding boiled potato juice.

Some ordinary potato juice was boiled in a small Erlenmeyer flask for 2–3 minutes, cooled and filtered. When tested with a tyrosine solution, it did not give the characteristic tyrosinase reaction, showing that the enzyme present had been destroyed.

A. 300 cc. phosphate buffered 0·05 % tyrosine solution (p_H 8·0) + 15 cc. potato juice + 30 cc. H_2O + 10 cc. toluene.

B. 300 cc. phosphate buffered 0·05 % tyrosine solution (p_H 8·0) + 15 cc. potato juice + 30 cc. boiled juice + 10 cc. toluene.

Control *A.* 300 cc. phosphate buffered water (p_H 8·0) + 15 cc. potato juice + 30 cc. H_2O + 10 cc. toluene.

„ *B.* 300 cc. phosphate buffered water (p_H 8·0) + 15 cc. potato juice + 30 cc. boiled juice + 10 cc. toluene.

Results.

Tyrosine present in g. per 330 *cc.* (*Average of two estimations.*)

	1	2	3	4	5
A. Ordinary potato juice	0·1427	0·1269	0·1062	0·0905	0·0321
B. Potato juice and boiled potato juice	0·1442	0·1185	0·0980	0·0804	0·0287
Time (mins.)	0	130	220	360	1380

The comparison is easier if the amount of tyrosine oxidised is represented as a percentage of the amount originally present, *i.e.* % of tyrosine oxidised.

	1	2	3	4	5
A.	0	11·08	25·58	36·59	77·50
B.	0	17·82	32·03	44·24	80·09

Average velocity coefficient for $A = 0{\cdot}00114$.
„ „ $B = 0{\cdot}00151$.

This activation has been noticed by Haehn [1919] who used boiled potato juice and tyrosine freed from the non-colloidal matter of potato juice by ultrafiltration, but this author erroneously attributes it to the presence of certain salts (calcium salts, phosphates, etc.) in the potato juice which act as co-enzymes. This activator or co-enzyme is not an ordinary inorganic salt, but is probably of organic nature, since it is not present in the ash of boiled potato juice, as shown below. It is interesting to note in this connection, that this activator is nearly always present in the boiled juice of new potatoes, but rarely, if ever, present in that from old potatoes.

Effect of adding the ash of boiled potato juice.

Boiled potato juice containing the activating agent referred to in the previous experiment, but having no tyrosinase reaction, was evaporated down to dryness and ashed. The ash was dissolved in a little warm dilute HCl, neutralised and made up to the original volume, a precipitate of the hydroxides of aluminium and iron remaining in suspension. To prove the activating power of the original boiled potato juice, an experiment was included to show that the oxidation of tyrosine by the unboiled potato juice used was accelerated by the addition of some of this boiled juice.

Solution *A.* 300 cc. tyrosine solution (buffered at p_H 8·0) + 15 cc. potato juice + 30 cc. H_2O + 10 cc. toluene.

„ *B.* 300 cc. tyrosine solution (buffered at p_H 8·0) + 15 cc. potato juice + 30 cc. boiled juice + 10 cc. toluene.

„ *C.* 300 cc. tyrosine solution (buffered at p_H 8·0) + 15 cc. potato juice + 30 cc. solution of ashed juice + 10 cc. toluene.

Controls *D*, *E* and *F* for solutions *A*, *B* and *C* respectively were set up under exactly the same conditions, except that the buffered solutions in these cases contained no tyrosine.

Results.

Time (mins.)	0	128	243	385							
	Tyrosine (g.) present in 330 cc. of solution. Average of 2 estimations				% tyrosine oxidised			Velocity coefficients			
	1	2	3	4	2	3	4	2	3	4	Average
A. (normal)	0·1415	0·1237	0·1050	0·0878	12·58	25·80	37·95	0·0011	0·0012	0·0012	0·0012
B. (+boiled juice)	0·1415	0·1144	0·0938	0·0747	19·19	33·69	47·23	0·0017	0·0017	0·0017	0·0017
C. (+ashed boiled juice)	0·1415	0·1248	0·1066	0·0896	11·73	24·64	36·67	0·0010	0·0012	0·0012	0·0011

Summary and conclusions.

1. Tyrosinase first produces from tyrosine a red substance. This process requires the presence of the enzyme and only takes place in the presence of oxygen. This red substance becomes colourless spontaneously and is then oxidised to form melanin. These last two processes take place in the absence of tyrosinase, but may be accelerated by it or by other oxidases found in potato juice.

2. The hydrion concentration of the medium has a marked influence on the velocity of the oxidation of tyrosine by tyrosinase and an even greater influence on the nature of the pigments produced. The limits of p_H between which potato tyrosinase acts upon tyrosine are 5 and 10. In the neutral and acid solutions, the main product during the first six hours is the red substance, but in alkaline solutions, the conversion of this into melanin is so rapid that the solutions quickly darken, and the preliminary reddening is not very marked.

3. Evidence is presented which is opposed to the view of Bach that tyrosinase contains a hydrolytic oxidation catalyst.

4. Haehn's experiment showing that tyrosinase, which has been rendered relatively inactive by dialysis, can be activated by the addition of certain mineral salts has been confirmed. But the acceleration of the enzyme action brought about by the addition of boiled potato juice is not due to inorganic salts but to some organic constituent or constituents of the potato juice. This activator is not always present in boiled potato juice, but is usually to be found in the boiled juice of new potatoes.

5. In order to obtain any true comparison between the oxidations of tyrosine by tyrosinase under different conditions, it is necessary to have all solutions well buffered at a definite hydrion concentration. In addition, the temperature must be kept constant and a satisfactory supply of oxygen must be maintained.

6. The results obtained show that the reaction is of the uni-molecular type, giving a true logarithmic curve when values of the concentration of tyrosine are plotted against the time, and also giving values for the velocity coefficient, which are approximately constant throughout the reaction. Tyrosinase is thus a true catalyst.

7. The oxidation of tyrosine by tyrosinase in a buffered solution at p_H 8·0 has a greater velocity than that at p_H 7·0, and the velocity at p_H 7·0 is greater than that at p_H 6·0.

8. The acceleration of the enzyme action by the addition of boiled potato juice has been definitely proved by following the rate of disappearance of tyrosine and it has also been shown that the ash of the same boiled juice produces no acceleration of the action.

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LVI. THE ISOLATION FROM CABBAGE LEAVES OF A CARBOHYDRATE, HITHERTO UNDESCRIBED, CONTAINING THREE CARBON ATOMS.

By HAROLD WILLIAM BUSTON
(*Beit Scientific Research Fellow*)
AND SAMUEL BARNETT SCHRYVER.
From the Biochemical Department, Imperial College of Science and Technology, S.W. 7.

(*Received June 6th, 1923.*)

THE substance described in the following paper was obtained during the investigation of the water-soluble constituents of cabbage leaves. The main object of the research was the isolation of the simpler nitrogenous substances present, but non-nitrogenous substances obtained were also examined, it being thought that these might throw some light on the derivation of the nitrogenous bodies.

During the course of the work, the method for separation of amino-acids and peptides from sugars by the carbamate method [Buston and Schryver, 1921] has been elaborated and rendered more complete; fractions containing 8–12 % nitrogen have been produced, which await further investigation. Amongst non-nitrogenous substances isolated may be mentioned citric and mesaconic acids. The latter does not appear so far to have been isolated as a natural product, and its preparation from cabbages may throw some light on the formation of citric acid from glucose. These points will form the subject of a later communication.

The product described in this paper appears to be of sufficient interest to warrant a separate mention.

The method adopted was briefly as follows:

An ether-water extract of 90 kilos. of the minced leaves was obtained, and the protein present flocculated as described by Chibnall and Schryver [1921]. The clear solution was then precipitated successively with alcohol, phosphotungstic acid, and baryta-alcohol; finally the major part of the nitrogenous substances was removed by repeated treatments with baryta and carbon dioxide in the presence of alcohol, according to the method described by the authors [1921].

The liquid remaining after this treatment was treated with sulphuric acid to remove barium quantitatively, and evaporated to a syrup. The syrup was left standing for two or three days under three times its weight of 90 % alcohol, by which time a fair quantity of crystalline matter had separated. This was sucked off on the pump, and dried on a porous plate. 36 g. of pale brown, somewhat sticky crystals were obtained, containing 0·9 % nitrogen. By treating in a Soxhlet for six hours with absolute alcohol, the colouring

matter and nitrogenous substances were removed, leaving 25 g. of white crystalline material. Of this, 51 % was found to be inorganic matter, mainly potassium chloride. The crystals were boiled with glacial acetic acid and filtered hot; on cooling, a bulky crop of fine white needles separated. These were removed and dried on a plate; weight obtained, 9·5 g. The mother liquor, on evaporation, gave a further crop of 1·2 g. The crystals still contained small amounts of potassium chloride, but were freed from this by repeated recrystallisation from glacial acetic acid. The total yield was about 10 g. from 90 kilos. of leaves.

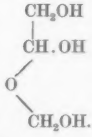
The substance was finally obtained as a white crystalline powder, readily soluble in water and in hot glacial acetic acid; but practically insoluble in alcohol and other organic solvents. The substance possessed a faint but perceptible sweetish taste. Its melting point was 148°.

The combustions gave the following figures:

C = 33·44, 33·19 %; H = 7·30, 7·49 %; O = 59·26, 59·32 %.

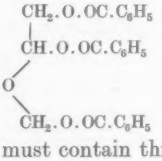
A molecular weight determination in aqueous solution by the freezing point method gave: M = 111·4. The above figures indicate the formula to be $C_3H_8O_4$. (This requires C = 33·34 %; H = 7·40 %; O = 59·26 %. Mol. wt. 108.)

The only constitutional formula to satisfy this would be that of a trihydroxy-ether:



To test this, it was decided to prepare the benzoyl derivative. 0·3 g. of the substance, treated in the ordinary way with benzoyl chloride and alkali, and extracted with ether, gave, on evaporation of the ether solution, 1·3 g. of a colourless, almost solid syrup. This was granulated with ligroin and could then be recrystallised from benzene. 0·7 g. white crystals were thus obtained; M.P. 52°–53°. Combustion of this gave: C = 68·20 %, H = 5·11 %, O = 26·69 %. Molecular weight in benzene solution, 434. (The tri-benzoyl derivative requires C. 68·58 %; H = 4·78 %; O = 26·68 %. Mol. wt. 420.)

The benzoyl derivative must therefore contain three benzoyl groups, and have the formula:

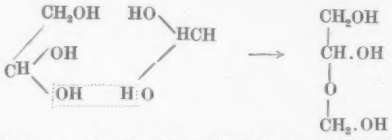


So that the original substance must contain three hydroxy groups, and have the formula given above.

Examination of this formula will show that the central carbon atom is asymmetric; the substance obtained, however, was optically inactive. This

may have been due to racemisation produced by the hot glacial acetic acid, or by the long contact with baryta in the earlier stages of separation.

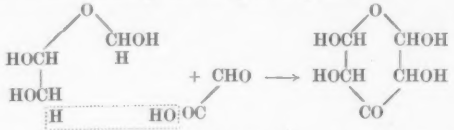
The formula also suggests the derivation of the substance by the condensation of formaldehyde and glycollic aldehyde (which may itself be regarded as the first condensation product of formaldehyde), reacting in the hydrated form:



It might thus be regarded as a simple disaccharide, glycollic aldehyde and formaldehyde being regarded as diose and monose respectively. Its formation from this source would thus make it appear to be formed as an alternative to a triose.

Various experiments were carried out to test for disaccharide-like properties in the substance. It showed no reducing properties, and gave no osazone. After heating with 5–10 % hydrochloric acid for some time, the neutralised solution still did not reduce Fehling's solution, though it reduced permanganate on warming. No formaldehyde could be detected. The substance therefore differs from normal disaccharides in its great stability (resembling more the ethers, in which class its formula also places it). Possibly the smallness of the molecule is the cause of its stability.

It is interesting to note further that by condensation with a molecule of glyoxylic acid a pyrone ring, which forms the basis of many naturally occurring substances such as the anthocyans, might be produced:



Various similar condensations of the substances or its derivatives could be suggested.

Summary.

An account is given of the isolation from cabbage leaves of a carbohydrate hitherto undescribed, of the formula $C_3H_8O_4$. This crystallises in needles, M.P. 148° which are readily soluble in cold water and hot glacial acetic acid, but insoluble in most other organic solvents. It yields a tri-benzoyl derivative, formula $C_3H_5O_4(C_6H_5CO)_3$; M.P. 52°–53°. The carbohydrate therefore has the constitution $CH_2(OH) . CH(OH) . O . CH_2(OH)$.

A possible mode of formation from formaldehyde in the plant is outlined, and it is suggested that it may form a stage in the making of a pyrone ring, which is an important constituent of the molecules of the anthocyans.

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Buston and Schryver (1921). *Biochem. J.* **15**, 636.
Chibnall and Schryver (1921). *Biochem. J.* **15**, 60.

LVII. INVESTIGATIONS ON GELATIN. PART II. RESEARCHES ON THE METHODS OF PURIFYING GELATIN.

By JOHN KNAGGS, ALEXANDER BERNARD MANNING and SAMUEL BARNETT SCHRYVER.

From the Biochemical Department, Imperial College of Science and Technology.

Report to the Adhesives Committee of the Department of Scientific and Industrial Research.

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INTRODUCTION.

In spite of the fact that a very large literature exists dealing with the physical and chemical properties of gelatin, no definite criterion seems to have been established up to the present time by means of which the purity of the material investigated can be defined. The necessity for defining the state of purity is an urgent one, when the origin of a sample of gelatin is considered. In the first place, it is derived from animal tissues, which, in addition to the actual precursors of gelatin, contain other complex nitrogenous substances, which may contaminate the finished product, and in the second place, it is itself produced from these precursors by the chemical action of hot water, undergoing during the process degradation into simpler products, the nature of which has not yet been definitely established. It follows, therefore, that every sample of gelatin is a mixture of gelatin with other nitrogenous substances, even when the greatest care has been taken in its preparation.

The following investigations were undertaken with two main objects in view. It was desired in the first instance to establish some physical criterion, by means of which the state of purity of a given sample could be defined; in the second instance it was necessary to discover processes by means of which the nitrogenous contaminations of gelatin could be removed, so as to yield a product conforming with the established physical criterion, which should remain unchanged when these processes were repeated an indefinite number of times.

An ordinary high grade commercial preparation, when allowed to soak in water, yields to the aqueous phase certain amounts of diffusible nitrogenous substances, the amounts of which vary from preparation to preparation, when the same conditions of experiment are chosen. These substances comprise, for the most part, the contaminating substances to which reference has been

already made. If a gelatin preparation is free from these, then a definite equilibrium of some kind should exist between the nitrogenous contents of an aqueous phase and a gelatin phase (either in the form of a jelly or otherwise) which are in contact with one another. If this equilibrium can be ascertained, then a criterion for the definition of the state of purity of a sample of gelatin will have been established.

As a result of the investigations described in this communication, preparations of gelatin have been obtained in which the desired nitrogenous equilibrium has been found to exist, and which must be taken, therefore, to be free from diffusible nitrogenous contaminations.

The researches started with the determination of the amount of nitrogen which diffused out from a given surface of a gelatin jelly, prepared under certain specified conditions, into a given volume of water in contact with it within 20 hours at the temperature of 20°. This method of investigation yielded valuable information as to the character of commercial samples of gelatin and glues (to which reference has been made in the *Bulletin on Adhesives* issued by the Department of Scientific and Industrial Research in 1922) and the determination of the so-called "diffusible nitrogen" is suggested as a routine method for the examination of these products. If the water in contact with the jelly is removed daily, and the nitrogen is estimated in each portion, it will be found that this diminishes rapidly until it reaches a small quantity, which remains constant over a prolonged period. Similar experiments were carried out with relatively large amounts of gelatin jelly with large surfaces in contact with water, and it was found that even after several months of contact, the amount of nitrogen in the water phase was continually increasing. The experiments illustrating these statements are given in Section I of this paper.

A detailed examination was then undertaken of certain properties of the nitrogenous diffusate, and also of a solution of gelatin (10 %) which had been heated for several hours, as it was assumed that the diffusate consisted largely of the products of thorough decomposition of gelatin. When these products were submitted to dialysis through parchment paper, it was found that at first relatively large amounts of nitrogenous substances diffused through in one day, but these somewhat rapidly diminished, until, after a certain time, the quantities passing the dialyser in a day became practically constant. This apparent constancy was maintained for several weeks. A diminution of the rate of diffusion could then only be ascertained when the diffusate of several days was estimated together. The rate of diffusion was so slow, that the results originally suggested that there were products present within the dialysers which were undergoing continual degradation in the presence of water at ordinary room temperature. These results are described in Section II.

The general result of the experiments given in Sections I and II was to indicate the great difficulty, if not the impossibility of freeing gelatin from the nitrogenous substances contaminating it by the method of washing or dialysis.

Incidentally, an examination was made of the products which passed the dialysers and those which remained within. The differences in the "Hausmann numbers" indicate that gelatin on heating does not break down into smaller aggregates of the same chemical character. The nature of the thermal decomposition of gelatin has not yet been definitely established.

As it had been shown that gelatin could not be freed from the contaminating nitrogenous substances even by months of continuous washing and dialysis, the effect of electrolysis on their removal was next investigated. It was found that by this means simple electrolytes such as salts were readily removed. Electrolytes of colloidal character and non-electrolytes remained, however, in the gelatin, when submitted to electrolysis. A simple ampholyte, such as glycine, which has weak acidic and basic dissociation constants, was removed only very slowly under the influence of an electric current. The result of these experiments, which are described in Section III, was to indicate, that, even by electrolysis, the nitrogenous contaminating products could not be completely removed.

Electrolysis, however, removes very readily the last traces of salts and other strong electrolytes from gelatin, which can thus be obtained practically ash-free. The electrolysis process, as described in this paper, is best applied to gelatin, from which the greater part of the salts have been already removed by washing the jellies with dilute acid and water.

When freed from electrolytes, gelatin possesses the property of separating out as a hydrated insoluble precipitate when solutions of 2 % concentration or less are cooled. Advantage has been taken of this fact to purify gelatin still further by a process conveniently termed "recrystallisation." The nitrogen content of the clear liquid after separation of the insoluble hydrate has been determined after successive "recrystallisations." This was found to fall to a certain constant value which remained the same whether the gelatin separated from a concentration of 0·1 or 2 %. Such a condition should hold when a substance is in equilibrium with its saturated solution, and the actual experiments indicate that such a saturated solution contains about 0·056 % of gelatin[1]. This result is in close accord with that found by Fairbrother and Swan [1922] although obtained by an entirely different method. Some of the results of these investigators are not, however, in agreement with those obtained by the authors of the present paper. It has been found impossible to obtain nitrogenous equilibrium between the aqueous phase and the gelatin phase in the short time suggested by Fairbrother and Swan; such an equilibrium was not attained with a well-washed gelatin sample even after several months. The gelatin was estimated by those authors in the aqueous phase by precipitation with tannic acid. This reagent will, however, precipitate, in addition to gelatin, a great part of the degradation products which are present as contaminants. The method employed by Fairbrother and Swan appears to the authors of this paper to be affected by two sources of error, which will

[1] See however, p. 486.

tend to compensate one another, and the accordance found in the determination of the solubility of gelatin in water by the two sets of investigators must be to a large extent accidental. The experiments on the "recrystallisation" of gelatin are described in Section IV.

According to the researches herein described, gelatin free from soluble nitrogenous contaminants can be obtained by submitting it to the following successive processes: (i) washing the jelly first with weak acid and then with water, (ii) submitting the washed concentrated jelly to electrolysis, and (iii) recrystallising the electrolysed gelatin.

Certain samples of gelatin thus purified still appear to contain a very small amount of an insoluble nitrogenous contaminant, which imparts to the jellies an opalescent appearance. Investigations on the separation of this are still in progress. Some gelatins appear to be nearly free from it. It is, however, only present in very small quantities and does not influence to any large degree the properties of gelatin, as do the water-soluble contaminants.

EXPERIMENTAL.

Section I. The "Diffusible Nitrogen."

When gelatin is placed in contact with water, a certain amount of nitrogenous material passes out from it. If the experiment is carried out under conditions defined as regards strength of gelatin, volume of gelatin and water, area of contact of phases, time of contact and temperature, the number of milligrams of nitrogen which pass into solution is termed the "diffusible nitrogen."

The conditions employed in the experiments described below were as follows: 20 g. of gelatin and 100 cc. of water were placed in a wide-mouthed stoppered bottle of $9\frac{1}{2}$ cm. internal diameter. The gelatin was dissolved in the water at 37°, and the solution allowed to set for 24 hours at room temperature. 100 cc. of water were then poured gently on to the surface of the gelatin, and the bottle was placed in a thermostat at 20° for 24 hours. The water was then poured off through a filter, and the surface of the gelatin and sides of the bottle were washed once or twice with distilled water, which was also poured through the filter. The nitrogen in the filtrate was then determined. (The "diffusible nitrogen.") In nearly all the experiments described in this paper, Coignet's "gold-label" gelatin was employed. In a few experiments other samples of gelatin were also occasionally employed, and these yielded the same results as Coignet's. For the latter, the "diffusible nitrogen," determined under the conditions mentioned above, gave values varying from 10–20 mg. If, after the first determination of the diffusible nitrogen, the process is repeated on the same gelatin by the addition each time of 100 cc. of water, the value falls to 2 mg. per 24 hours, at which it remains sensibly constant (with occasionally higher values, owing to the swelling and increase

of surface of the gelatin). As an illustration, the results of one experiment are given below:

"Diffusible nitrogen" in Coignet's "gold-label" gelatin.

	Period of contact	Diffusible nitrogen mg.	Diffusible nitrogen in 24 hours mg.
1.	24 hours	11·6	11·6
2.	24 ,,	5·8	5·8
3.	48 ,,	8·65	4·32
4.	48 ,,	7·01	3·50
5.	48 ,,	6·46	3·23
6.	72 ,,	9·16	3·05
7.	8 days	20·6	2·57
8.	72 hours	9·37	3·12

Fresh water was used in each experiment.

The process cannot be carried on indefinitely, since the gelatin ultimately swells and the surface breaks up. If the gelatin is washed until the approximately constant nitrogen value is reached, and then remelted at 37°, and allowed to set, it is found that the "diffusible nitrogen" has risen again nearly to its original value. For example, a sample giving the initial value of 15·3 was washed till the value fell to 1·96. On remelting it rose to 12·1. It appears probable that the diffusible nitrogen comes from the surface layer or its immediate neighbourhood only. This statement is confirmed by experiments in which different areas of contact between jelly and water were used, the other conditions being exactly similar. Thus, when the ratio of the two surfaces was as 2·2:1, the diffusible nitrogens were 27·2 and 14.

The ultimate small value (2 to 3) obtained by continuous washing may be due to (*a*) simple solubility, (*b*) slow diffusion from the lower layers, (*c*) slow degradation of the gelatin in contact with water. The "diffusible nitrogen" is not exactly proportional to the concentration of the gelatin. Thus

Concentration of gelatin	"Diffusible nitrogen"
5 g. gelatin + 100 cc. water	7·4
10 ,, ,,	10·0
20 ,, ,,	12·6

Washing the gelatin with dilute hydrochloric acid and then with water (Loeb) produced a marked lowering of the diffusible nitrogen; thus, for example:

First	24 hours.	Diffusible	nitrogen	2·5
Second	,,	,,	,,	1·3
Third	,,	,,	,,	1·25

Fresh water was used each time.

This case was somewhat exceptional, other samples giving the higher values of 4 to 6 for the first 24 hours.

The use of very dilute hydrochloric acid ($N/1000$ or less) for making up the gelatin and washing it makes no appreciable difference to the diffusible

nitrogen. The presence of neutral salts, on the other hand, increases it, as the following experiments (some of which were carried out by Mrs Horne) show:

Salt used	Concentration of salt in jelly and water	First four days	Next six days	Next eight days
NaCl	0·0	15·3	12·3	17·4
	N/10	18·6	14·7	20·3
	N/4	19·2	15·8	21·7
	N/2	21·9	18·2	23·3
$CaCl_2$	0·0	17·4	15·6	16·5
	N/10	21·5	19·4	19·7
	N/4	24·9	—	19·5
	N/2	25·8	—	20·3

Fresh salt solutions were employed for each of the periods of experiment given in the above table.

The "diffusible nitrogen" is derived to a large extent, if not wholly, from the products of thermal degradation of gelatin, as is shown from the following experiment. A 20 % gelatin was made up in a series of wide-mouthed bottles, as described above, and each bottle was heated for a given period in a hot-water oven. After removal, the gelatin was allowed to set for at least 24 hours, and the "diffusible nitrogen" determined in the usual manner. The results are indicated in the accompanying table and curve (Fig. 1). After 16 hours the jelly is so weak, that the determination of the diffusible nitrogen is unreliable. After 29 hours' heating the gelatin would not set at all. The curve for the first 16 hours is a regular one.

The experiments were carried out with a gelatin that had been well-washed beforehand.

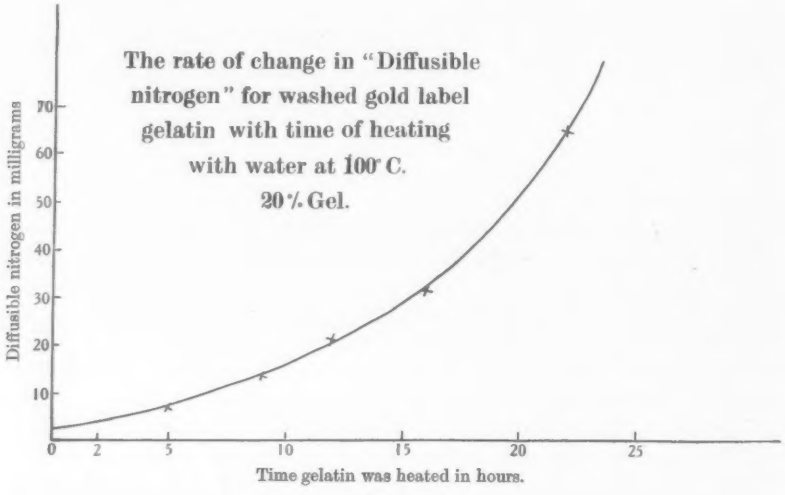

Fig. 1

	Diffusible nitrogen
Unheated gelatin	1·9
Heated 5 hours	6·9
„ 9 „	13·1
„ 12 „	21·4
„ 16 „	32·6
„ 22 „	59·9
„ 29 „	Gelatin did not set

The question next arose as to whether, on leaving gelatin a sufficiently long time in contact with water, an equilibrium value for the nitrogen content of the water could be reached. To test this 112 g. of gelatin, previously washed carefully with dilute hydrochloric acid and water, were immersed in the form of thin sheets in 3 litres of water, a little chloroform having been added to keep the gelatin sterile. The whole was kept in a tightly stoppered bottle and left for prolonged periods during which, at intervals, 50 to 100 cc. of the aqueous phase were removed, filtered and submitted to an estimation of nitrogen. No indication of an equilibrium was ever found, as the following numbers indicate:

Period of contact	Milligrams of nitrogen per 100 cc. of aqueous phase
2 months	43·0
3 „	45·5
4 „	48·9

The water after this time was poured off, the gelatin was washed, and fresh water was added. The nitrogen in portions of 50–100 cc. was determined at intervals with the following results:

Period of contact	Milligrams of N per 100 cc. aqueous phase
7 days	11·2
14 „	12·6
21 „	14·6
28 „	16·0
53 „	20·3
4 months	35·3

Thus, after even 8 months of washing, nitrogen appeared to be still coming out from the gelatin into the water. These results are at variance with those of Fairbrother and Swan [1922]. They suggest that gelatin itself undergoes some degradation in contact with water. It was therefore considered advisable to obtain some idea as to the diffusibility of the nitrogenous products which could be washed out of the gelatin, and experiments dealing with this factor are given in the next section.

Section II. The diffusibility through parchment of the nitrogenous products which can be extracted from gelatin by water.

Two main series of experiments were carried out. (*a*) With the washings of gelatin. (*b*) With a sample of gelatin, which had been previously well washed till nearly salt-free and then heated for a long period, so that it no longer set in concentrated solutions.

(*a*) *Experiment with the washings.* The following experiments carried out by Mrs Horne, were made with a sample of Nelson Dale and Co.'s bone gelatin, which was kindly provided by Mr W. K. Beveridge. Similar results were got with Coignet's "gold-label" gelatin.

Three extracts (I, II and III) were made. Extract I was made by extracting 2 lbs. of gelatin first with 10 litres of water for 4 days, then after pouring off the water which was not absorbed by the gelatin, and which amounted to about $2\frac{1}{2}$ litres, with 10 litres of water for 2 days, this extraction being followed by another extraction by 10 litres of water for 2 days. The washings from these extractions, which lasted altogether for 8 days, were combined to form extract I.

The second extract (II) was made by treating the washed gelatin with 10 litres of water for 13 days, whilst extract III was made by a subsequent extraction with the same amount of water for 24 days. The three extracts were concentrated under diminished pressure until they all contained the same amount of nitrogen, viz. 246·6 mg. per 100 cc. The concentrated solutions were then introduced into parchment dialysers and submitted to dialysis under as nearly as possible identical conditions, *i.e.* the size of the parchment bags, the size of the containing vessels, the amounts introduced into the dialysers, and the water outside were the same in all cases. All the experiments were carried out under bell-jars under which were placed also open dishes containing chloroform so as to keep the solutions sterile throughout the whole course of the experiments. 144 cc. of solution were introduced into the dialysers and 600 cc. of water were placed outside. The exterior water was changed from time to time and the nitrogen content thereof estimated. The period of dialysis into the same exterior liquid (600 cc.) is indicated in the second column.

Rate of dialysis of nitrogenous substance from three washings of the same portion of gelatin.

Time from commencement of experiment Days	Period of dialysis Hours	Extract I mg. N	Extract II mg. N	Extract III mg. N
1	24	29·07	16·88	13·45
2	,,	15·01	9·2	10·26
3	,,	10·53	5·93	6·21
4	,,	9·09	5·64	4·56
7	72	16·5	8·25	9·22
8	24	5·65	3·36	3·06
9	,,	4·95	2·83	2·74
10	,,	5·54	3·4	—
11	,,	4·49	2·94	—
14	72	9·9	6·6	—
15	24	4·04	3·36	—
16	,,	4·04	3·25	2·6
17	,,	—	3·2	2·59
18	,,	4·34	3·28	2·59
24	144	15·5	11·3	8·89
30	,,	14·2	11·84	—
36	,,	12·28	9·95	7·2
42	,,	7·79	7·22	5·88

It will be noticed from the above table that even the third extract of the gelatin, after as long an interval of dialysis as 6 weeks, still contains nitrogenous substances which can pass the dialyser, the amount of nitrogen thus passing amounting to the quite appreciable quantity of nearly 1 mg. per day. About 30 % of the nitrogen of extract I and 18 % of extract II had diffused through the parchment in 14 days.

(*b*) *Experiment with heated gelatin solution.* 200 g. of gelatin, which had been previously washed till it was nearly ash-free, were dissolved in 4 litres of water, and the solution was heated for 48 hours in an enamelled iron digester with a reflux condenser in a glycerol bath at 110°. A dark-coloured insoluble compound was formed, which was filtered off. The filtrate was concentrated to a thick syrup under reduced pressure at 40°. This syrup was then dissolved in water and 200 cc. of a solution containing 1200 mg. of nitrogen were introduced into a parchment dialyser. The water on the outside of the dialyser was 500 cc. and this was changed daily and the nitrogen estimated therein. The results are given in the accompanying table. The second column of the table gives the amount of nitrogen in each sample of the dialysate, and the third column the total amount which had diffused through after the period of dialysis indicated in the first column. The rate of dialysis is rapid at first, but quickly diminishes, till after about 21 days it becomes constant at 7 mg. a day, which is equivalent to 0·58 % of the total of the nitrogen originally in the dialyser and 1·6 % of that in the dialyser at this period. These experiments were continued for 60 days without any apparent diminution in the daily rate. After 3 months, however, when the exterior fluid was changed only once every 6 days, a small diminution of the rate of dialysis could be detected. The rate of diffusion is indicated on the curve (Fig. 2). This, it will be observed, becomes practically a straight line after 20 days. These experiments indicate quite clearly the intensely slow rate of diffusion of the degradation products of gelatin, and the impracticability of freeing gelatin from them by any process of washing or dialysis.

The results for the rate of diffusion through parchment of the sample of Coignet's Gold Label gelatin (ash-free) which had been heated for 48 *hours at* 100°.

Time Days	Quantity of nitrogen diffused in mg.	Total amount of nitrogen diffused in mg.	Percentage of nitrogen diffused through parchment
1	116·0	116·0	9·68
2	68·2	184·2	15·4
3	40·4	224·6	18·6
4	35·5	260·1	21·6
5	29·3	289·4	24·0
6	—	—	—
7	43·1	332·5	27·6
8	22·5	355·0	29·6
9	22·0	377·0	31·4
10	20·3	397·3	33·0
11	18·6	415·9	34·6
12	18·9	434·8	36·2
13	—	—	—
14	24·6	459·4	38·2
15	14·4	473·8	39·5
16	15·0	488·8	40·6
17	9·5	498·3	41·5
18	8·6	506·9	42·2
19	8·5	515·4	42·9
20	—	—	—
21	12·6	528·0	43·8
22	7·8	535·8	44·5
23	7·7	543·5	45·2
24	7·8	551·3	45·9
25	7·8	559·1	46·4
26	7·8	566·9	47·2

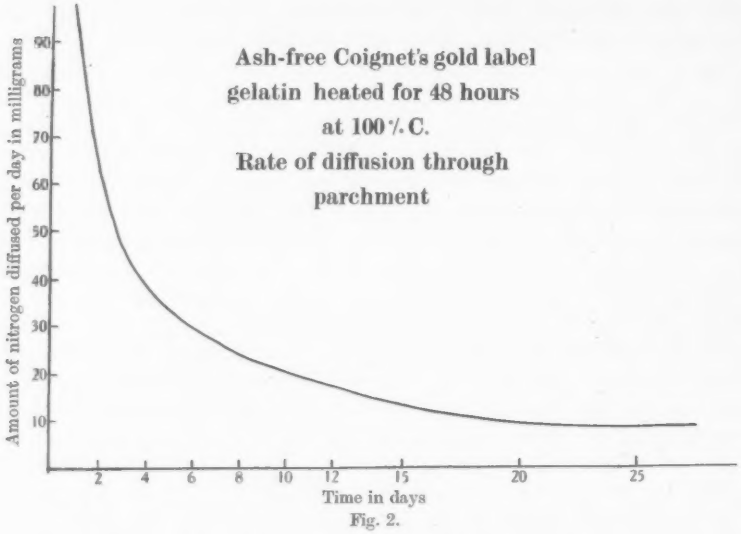

Fig. 2.

A few experiments were carried out with the object of throwing some light on the nature of the thermal degradation of gelatin. No very definite scission of the peptide linkage could be ascertained by either the formalin titration method, or by the estimation of the nitrogen set free by nitrous acid by the method of van Slyke. (There were certain difficulties in applying this method, which will not be discussed in this place.) It was thought that heat might bring about a simple disaggregation of a large molecule into simpler aggregates of the same type. If this were the case, the part of the degraded gelatin which passes the dialyser should yield the same hydrolysis products as the part that remained within. This, however, was not found to be the case as the following determinations of the Hausmann numbers show:

	Amide N	Humin N	Diamino N	Humin + diamino N
Ash-free Coignet's "gold-label" gelatin	1·1	—	22·3	—
A. The same, of which a solution had been heated for 48 hours at 100°	1·2	2·2	21·5	23·5
Dialysis of A through parchment for 21 days:				
(i) Portion which diffused through	1·25	1·3	26·5	27·8
(ii) Portion which did not diffuse	1·1	2·6	20·5	23·1

The diamino-nitrogen of the diffusible nitrogen was distinctly higher than that of the non-diffusible.

Section III. Experiments on the purification of gelatin by electrolysis.

Loeb's method of purifying gelatin [1922], based on the consideration of its amphoteric character, consists in treating the material first with a solution of hydrochloric acid with a hydrion concentration just greater than that of iso-

electric gelatin and then with water until it is free from acid. It was found, however, that only by very prolonged treatment by this method was it possible to remove the last traces of electrolytes, and it was considered advisable to supplement it by a method of electrolysis. The presence of very small amounts of electrolytes modifies very considerably the properties of gelatin, and an efficient method for obtaining a gelatin which is practically ash-free is, therefore, very essential. It was also necessary to ascertain the efficiency of an electrolytic method for removing the nitrogenous contaminations. The apparatus employed is shown in the accompanying illustration (Fig. 3).

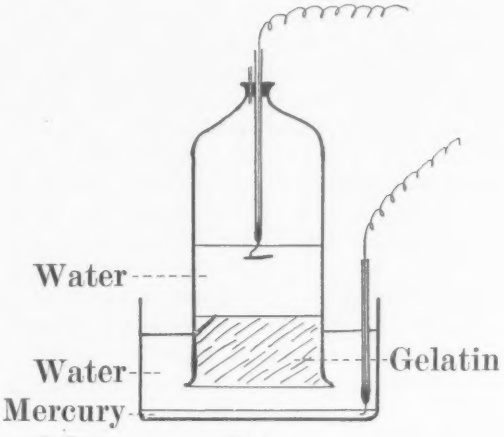

Fig. 3.

One to 1·5 litres of a gelatin solution of 10–20 % were allowed to set in the lower part of a bell-jar of 5 cm. diameter. Both the upper and lower surfaces of the gelatin were in contact with water and a current was passed through the system from a platinum anode to a mercury cathode. The distance from anode to cathode was about 20 cm. The initial voltage employed was usually 100 which was afterwards increased to a maximum of 220. The water was changed once a day at least, and the process was continued until there was no further appearance of alkali at the cathode or of acid at the anode. The gelatin was then removed and cut into slices which were dried in a current of filtered air at ordinary room temperature. The properties of the gelatin thus prepared are referred to in greater detail in the next section.

The gelatin used as the starting material in these experiments was Coignet's "gold-label." It had an ash content of about 1·5 %. The initial washings with dilute acid and water brought this down to 0·4–0·1 %, which was further reduced by the electrolysis to 0·02 % or less. It will be noticed that no membranes were employed for the process. This was found to be possible when the apparatus described above was employed, if the greater part of

the ash content had been first removed. If a sample containing the original amount of ash (1·5 %) was submitted to electrolysis by this method without any previous treatment, it was found that acid gelatin formed towards the anode and alkali gelatin towards the cathode. These underwent swelling, and particles of the jelly escaped into the water, and often the whole jelly broke away from the bell-jar. The method was quite satisfactory, however, for gelatin which had been submitted to a preliminary purification by acid.

Efficiency of the process of electrolysis. A number of experiments were carried out with a view to determine the efficiency of the electrolytic process in removing contaminations other than the inorganic.

The first experiments were made with aniline dyes, and these were carried out in a smaller apparatus than the one described above. This consisted of a simple glass tube of about 5 cm. diameter, which was used instead of a bell-jar; the remainder of the apparatus was practically the same. Into the tube were introduced 50 cc. of a solution of electrolyte-free gelatin (made up from 20 g. gelatin to 100 cc. water), to which the dye was added. The solution was then allowed to set. Methylene blue and methyl red were readily removed from the gelatin by electrolysis, the former to the cathode, the latter to the anode. The colloidal dyes, Congo-red and night-blue were, however, not removed, under the highest potential gradient available (about 25 volts/cm.). Sugars were not removed at all, whilst glycine, an ampholyte with both acid and basic dissociation constants very weak, was only removed very slowly.

Experiments were also carried out to determine the rate of removal of the ordinary nitrogenous contaminants of gelatin. Two 50 cc. portions of an electrolyte-free gelatin (20 g. gelatin + 100 cc. water) were introduced into tubes 5 cm. diameter. One was submitted to electrolysis under a P.D. 60 volts with a distance of 8 cm. between the electrodes. The other tube, used as a control, was not submitted to electrolysis. From time to time the water round the electrodes was removed, and the nitrogen therein estimated. From the results, which are given in the following table, it will be seen that more nitrogenous matter passes out from the electrolysed than from the non-electrolysed gelatin. The experiment indicates the difficulty of complete removal of the nitrogenous contaminants by electrolysis.

	Electrolysed gelatin		Control	
	+	−	+	−
First 4 days	28·3	34·0	4·8	4·6 mg. N
Next 4 „	9·3	11·3	3·0	2·8 „
„ 5 „	6·1	6·5	2·4	2·6 „
„ 5 „	5·1	5·8	2·0	2·3 „
„ 10 „	6·3	6·5	3·5	4·0 „

The electrolyte-free gelatin was found to be extremely resistant to putrefaction.

Section IV. The "recrystallisation" of gelatin.

A gelatin, which had been purified by the washing and electrolytic processes described above, was found to have properties markedly different from the unpurified. A solution of 2 % or less of the air-dried purified material (which corresponds to about 1·6 % when calculated on the true dry weight) is clear at 40°, but on cooling becomes white and opaque. The gel or solution formed is unstable and on standing for 24–48 hours, the hydrated gelatin separates out in the form of a bulky precipitate, which can be separated from the clear supernatant fluid by centrifuging in the case of the more concentrated solutions or by simple filtration in that of the more dilute solutions. The "recrystallisation" of gelatin, if so it may be termed, can be repeated an indefinite number of times, and results in a further purification of the gelatin.

Solutions of 3 % and higher concentrations set to stable gels on cooling. The 3 % gel is white and opaque, but with increasing concentrations the gels become more and more transparent. The gels of 10 % strength and higher, are, as regards transparency, indistinguishable from those made from ordinary commercial gelatin.

To investigate the recrystallisation process as a means of purification, the nitrogen content of the supernatant fluid obtained by centrifuging and filtering was investigated. The experiments were usually carried out with a litre of solution. After cooling this was generally allowed to stand at room temperature (14–16°) for two or three days, before centrifuging. From a litre of 2 % (air-dried) gelatin, 300 cc. of a clear fluid were usually obtained. After the first "recrystallisation" this contained nitrogen varying from 10–25 mg. per 100 cc. If the separated gelatin is redissolved at 40° and the bulk of the solution is made up to 1 litre, and allowed to cool, the separated clear solution has a nitrogen content of 9–11 mg. per 100 cc. Further "recrystallisations" fail to reduce this amount. Moreover, a solution which had been allowed to stand at room temperature for 6 weeks gave practically the same value for the nitrogen in the supernatant fluid as one which had been allowed to stand for only 48 hours (9·8 and 9·2 mg./100 cc. respectively). This result is in marked contrast with those found for unpurified gelatins.

Another quantity of the "recrystallised" gelatin was washed by decantation with large quantities of water, then separated by centrifuging and again made up to the original volume at 46°. After cooling, the nitrogen content of the supernatant fluid was found to be 10·4 mg./100 cc.

There appears, therefore, to be an equilibrium value of about 10 mg./100 cc. for the nitrogen content of the clear liquid which separates after melting and cooling. This value is not attained unless the solution is heated. When some of the precipitated gelatin was made up to the original volume with cold water without remelting and allowed to stand for 3 days with occasional shaking the supernatant fluid contained only 5·0 mg./100 cc.

When the gelatin is not melted the equilibrium value is only attained

slowly. The following experiments were carried out with a gelatin which had been purified by electrolysis, but not by recrystallisation. 40 g. of gelatin in sheets were allowed to soak in 1 litre of water. After $2\frac{1}{2}$ months, the aqueous phase contained 8·3 mg./100 cc. and after $4\frac{1}{2}$ months 16·2 mg./100 cc. In another case 20 g. gelatin as a 10 % gel was broken up finely and immersed in 800 cc. water. After 2 months the water contained 11·9 mg./100 cc. Experiments on similar lines are being carried out with "recrystallised" gelatin but are not yet completed.

In all the experiments mentioned above, dealing with the supernatant liquid from the "crystallised" gelatin, 2 % solutions of the air-dried material were used. In the following experiments gelatin was allowed to crystallise from solutions of varying strengths. From even the smallest concentrations used the gelatin separates as a hydrate. The amounts of nitrogen in the supernatant liquid are given in the following table.

Initial concentration	N content of clear fluid mg. N per 100 cc.
1·39	11·1
0·697	9·57
0·348	9·38
0·139	11·7

The initial concentrations are here based on the nitrogen determinations in the solutions, it being assumed that pure dry gelatin contains 18 % N. These experiments were not carried out in a thermostat, and the materials used in the determination of the nitrogen contained minute quantities of nitrogenous impurities for which allowance has not been made.

It seems, therefore, that purified gelatin has a real but small solubility in water at the temperatures 14–16° corresponding to about 10 mg./100 cc. N or 0·056 % gelatin.

Unfortunately, further experiment throws some doubt on this explanation of the nature of the apparent equilibrium observed. The filtrate from a solution of the "recrystallised" gelatin was compared with a solution of the gelatin made up directly to the same nitrogen content. On the assumption that we are dealing with a true solubility phenomenon these solutions should be identical. Their viscosity, surface tension and diffusibility through parchment were determined and, within the limits of experimental error, were found to be the same for both solutions.

A litre of each solution was then concentrated under reduced pressure at 40° until the volume was brought to 200 cc. These solutions, which should now correspond to a 0·28 % solution of gelatin, were cooled. *No separation of solid gelatin* occurred from the concentrated filtrate, which was only slightly turbid, whereas from the solution made up directly gelatin separated as from an ordinary 0·28 % solution. This failure to reverse the process and obtain the solid hydrated gelatin from the filtrate on concentration throws doubt not only on the hypothesis of a simple solubility effect but also on the assumption that the results recorded in Section IV above represent an equilibrium. It is

difficult to conceive of any true equilibrium, whether based on solubility, adsorption or disaggregation effects, which would not be reversed by the procedure described. The non-reversibility is not due to non-appearance of the solid phase, since addition of small amounts of the solid does not affect it. In spite then of the agreement of the results of Section IV the existence of a definite equilibrium cannot yet be taken as proved. It is conceivable that gelatin is not a simple molecule but an aggregate. Its molecular weight as determined by Biltz was found to be about 17,000. Repetition of the determination by other methods in this laboratory gave numbers varying between 17,000 and 19,000. It is difficult to conceive that these numbers represent the weight of a simple molecule. The behaviour of such aggregates when dispersed in water cannot yet be predicted.

CONCLUSIONS.

(1) In ordinary high-grade commercial gelatin, the nitrogenous contaminations are not removed even by washing for several months.

(2) The nitrogenous contaminations, which are derived mostly from the thermal decomposition of gelatin during the course of its preparation, contain products which diffuse only very slowly. After several weeks of dialysis of the products obtained from gelatin by diffusion, or of the products obtained by heating purified gelatin in water, in a parchment membrane, diffusible matter is still found to be passing the parchment. The conclusion is drawn that it is practically impossible to free gelatin from its nitrogenous contaminants by washing or dialysis.

(3) It is difficult to free gelatin entirely from electrolytes by washing with acid and water by the method of Loeb. Gelatin purified in this manner, can, however, be readily freed from almost the last traces of stronger electrolytes by submission to electrolysis without the use of membranes. Electrolysis is not, however, efficient in removing substances of colloidal character and non-electrolytes or even weak electrolytes.

(4) Gelatin when purified by washing and electrolysis separates out from solutions of less than 2 % as an insoluble hydrate. When this process of "recrystallisation" is repeated, a gelatin is produced which separates from a supernatant fluid which always contains about 10 mg. per 100 cc. This quantity remains constant, from whatever strength of solution the gelatin separates. When a gelatin with this property is obtained, it indicates that it is free from the soluble nitrogenous contaminants. The gelatin which separates behaves in many respects like a pure substance in equilibrium with a saturated solution, which solution contains 0·056 % gelatin.

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LVIII. SOME NOTES ON THE DETERMINATION OF THE HAUSMANN NUMBERS OF PROTEINS.

By JOHN KNAGGS.

From the Biochemical Department, Imperial College of Science and Technology.

A Report to the Adhesives Committee of the Department of Scientific and Industrial Research.

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THE modification of Hausmann's method by Osborne and Harris [1903] does not give consistent results for gelatin as the following determinations made with three different samples show.

Gelatin	% diamino N
Coignet's Gold label	31·7
	20·8
	20·1
	19·7
Coignet's Gold label which had been washed with frequent changes of water for 10 days	29·9
	29·9
	19·6
Gelatin extracted from ossein at 100°	33·7
	20·3

The percentage of diamino nitrogen varies considerably, and as these results were determined in duplicate under the same conditions and the duplicates were found to give the same result it was concluded that the variations were due to the difference in treatment to which the hydrolysed product was subjected before precipitation.

On referring to the literature it was found that variable results were obtained by different workers.

	% diamino N
Hausmann (1899)	35·83
Van Slyke (1911)	25·5
Bogue (1920)	22·75
	21·68
	22·1
	26·47

These variations are far larger than can be accounted for by differences in the samples analysed and must be due to some faults inherent in the method.

Attempts were therefore made to ascertain the cause of these discrepancies. It was thought that it was due to the formation of a polypeptide under certain conditions. [Compare Dakin, 1920.]

It was quite conceivable that gelatin, in the presence of cold acid, which produces only incomplete hydrolysis, might form certain polypeptides which are only hydrolysed with difficulty.

Therefore experiments were carried out in which the gelatin was heated immediately on adding the acid, and after allowing the gelatin to stand in contact with the cold acid for 24 hours at laboratory temperature.

It was found that the percentage of diamino nitrogen was always higher when the gelatin had been allowed to stand with cold acid for a few hours as the following results show:

	Coignet's Gold label	Gelatin from ossein
Immediately hydrolysed for 12 hours	20·6	25·03
Hydrolysed for 12 hours after standing 24 hours at lab. temp.	24·25	35·62
" " " " 37°	23·7	34·65

It was noticed that where the results were high a secondary precipitate was formed after filtration of the phosphotungstates. This is probably due to the formation of some product of hydrolysis of gelatin which gave a precipitate with phosphotungstic acid which was somewhat soluble in water so that variations in the results were obtained with very slight variations in the conditions.

Experiments were then carried out to determine the rates at which peptide scission and formation of amino groups takes place, with gelatin hydrolysed immediately after mixture with the acid, and after standing with cold acid solutions. It was found that the maximum amount was formed in one hour in the first case and not until after 3 hours in the second case, indicating the formation of resistant groups.

	% hydrolysis	
	A	B
	Gelatin hydrolysed immediately	Gelatin hydrolysed after standing with acid at lab. temp. 24 hours
0 mins.	4·5	8·9
15 "	60·9	—
30 "	62·6	46·6
45 "	68·3	—
60 "	75·8	65·8
1·5 hrs.	75·8	—
2 "	75·8	70·6
3 "	75·4	74·8
6 "	75·7	75·1
28·5 "	75·6	75·0

The rate of hydrolysis was followed by Van Slyke's method for amino nitrogen.

In the case of A the percentage of diamino nitrogen was also determined at various intervals and it was found that although the amino nitrogen reached its maximum after 1 hour the diamino nitrogen did not reach a constant until 12 hours.

Time of hydrolysis in hours	% amino N	% diamino N
3	0·93	36·1*
6	1·0	25·7*
9	1·06	36·6*
12	1·1	20·05
16	1·1	20·09
20	1·1	25·1*†
24	1·1	20·0
28·5	1·23	20·1

* The filtrate from the phosphotungstate precipitate on standing gave a further precipitate.

† This result was abnormal as conditions were not observed which were found out later to have an effect on the results, *i.e.* the solution was allowed to stand overnight with the acid before precipitating.

It was also found that if the solution containing the diamino acids were allowed to stand some time with 5 % H_2SO_4 abnormal results were obtained.

Gelatin was hydrolysed for 30 hours with 20 % HCl. The amide nitrogen and humin nitrogen were removed. The filtrate was concentrated to 100 cc. and 5 g. H_2SO_4 added. The following results were obtained:

	% diamino N	Type of precipitate
1. Diamino acids precipitated immediately solution was cold	20·4	Granular and easily filtered
2. Diamino acids precipitated after allowing solution to stand 24 hours with 5 % H_2SO_4	23·9	Flocculent ppt. and secondary ppt. which separated after standing
3. Diamino acids precipitated after allowing solution to stand 48 hours with 5 % H_2SO_4	25·1	,, ,,
4. Same as 3 only diamino acids precipitated in warm solution and allowed to stand 24 hours before filtering	20·1	Granular

The results indicate that some change takes place when the amino acids are allowed to stand with acid in the cold. Probably a polypeptide is formed which is precipitated by phosphotungstic acid.

Experiments were carried out to prove the above. The gelatin was hydrolysed for 3 hours with 20 % HCl. After the amide and humin nitrogen had been removed the solution was made up to 100 cc. in 5 % H_2SO_4 and heated for varying lengths of time at 120° in the autoclave. The diamino acids were precipitated with 20 % phosphotungstic acid on cooling.

Method of precipitating diamino acids	% diamino N	Type of precipitate
1. Add 3 cc. H_2SO_4 and 30 cc. of 20 % phosphotungstic acid after removing amide and humin nitrogen	36·04	Gelatinous
2. Add 3 cc. H_2SO_4 and heat under reflux for 1 hour before precipitating	24·00	Flocculent
3. Add 3 cc. H_2SO_4 and heat in autoclave for 1 hour before precipitating	24·1	,,
4. Heat for 2 hours at 120°	23·25	,,
5. ,, 3 ,, ,,	20·5	Granular
6. ,, 4 ,, ,,	20·2	,,

These results indicate that the correct values for diamino nitrogen may be obtained by hydrolysing immediately for 3 hours, heating the solution with 5 % H_2SO_4 in the autoclave for 3 hours at 120° and precipitating the diamino acids immediately on cooling.

If the solution is allowed to stand a few hours before adding the phosphotungstic acid solution the precipitate will be flocculent or gelatinous instead of granular as the following show:

Gold label gelatin hydrolysed 3 hours with 30 % HCl

Method of precipitating diamino acids	% diamino N	Type of precipitate
1. Evaporate to 100 cc. add 3 cc. H_2SO_4 and 30 cc. of 20 % phosphotungstic acid	25·8	Flocculent
2. Evaporate to 100 cc. add 3 cc. H_2SO_4 and heat for 3 hours in autoclave at 120°	20·6	Granular
3. As 2 above, but allow solutions to stand 24 hours before precipitation with phosphotungstic acid	30·5	Flocculent with secondary gelatinous ppt.
4. As 2 above; allow to stand 4 days before ppt.	24·1	Flocculent
5. As 4 but filter immediately on adding phosphotungstic acid	20·4	—
6. As 4 above but heat to boiling before ppt.	20·7	Granular

The experiment was repeated with Coignet's "Gold Label Gelatin" which was allowed to stand with the acid 48 hours at laboratory temperature before hydrolysing for 12 hours.

Method of precipitating diamino acids	% diamino N	Type of precipitate
1. Ordinary way by acidifying solution to 5 % H_2SO_4 and ppt. with 30 cc. of 20 % phosphotungstic acid	24·5	Flocculent
2. Add 3 cc. H_2SO_4 and evaporate to 100 cc. Allow solution to stand 4 days then heat to 100° and when cold add the phosphotungstic acid solution	24·0	„
3. Evaporate to 100 cc. add 3 cc. H_2SO_4 and heat for 3 hours at 120° in autoclave and then ppt.	24·5	„
4. Evaporate to 100 cc., add 3 cc. H_2SO_4 and heat for 3 hours at 120° in autoclave. Allow to stand at lab. temp. 2 days before ppt.	30·9	Gelatinous ppt.

The latter results show that when the cold acid is allowed to be in contact with the gelatin for some hours before heating some product is formed which resists hydrolysis even after 12 hours with 20 % HCl and a subsequent heating with 5 % H_2SO_4 at 120° in the autoclave before precipitation of the diamino acids.

In all the above cases where a high value for the percentage of diamino nitrogen was obtained the precipitate formed with phosphotungstic acid was flocculent or gelatinous, and took some time to settle, whereas those which were normal gave a granular precipitate which settled quickly and was easily filtered.

To obtain a correct value for the percentage of diamino nitrogen in the hydrolysis products of gelatin the following precautions should be observed:

1. On mixing the protein and the acid for hydrolysis, the mixture should be heated to boiling almost immediately for 12 hours.

2. The solution which contains the diamino acids should not be allowed to stand any length of time before the phosphotungstic acid solution is added. If the solution has been standing with the H_2SO_4 it should be heated to boiling for a few minutes and when cool the diamino acids precipitated with 20 % phosphotungstic acid solution.

A correct result for the percentage of diamino nitrogen may be obtained by hydrolysing the gelatin for 3 hours with 20 % HCl at boiling point and, after removing the amide and humin nitrogen, adjusting the solution containing the diamino acids to 200 cc. and adding 3 cc. H_2SO_4. This solution should then be heated for 3 hours in the autoclave at 120° and when cool the phosphotungstic acid solution should be added. This method cannot be applied when the complete analysis is required seeing that the amide group is not entirely broken down until the gelatin has been hydrolysed with 20 % HCl for 12 hours.

All the difficulties encountered hitherto, appear to be due to the fact that the hydrolysis products condense in the presence of cold acids and these condensation products cause the precipitation of gelatinous phosphotungstates.

Experiments were made to find another precipitant for the diamino acids other than phosphotungstic acid. The following alkaloidal reagents were tried: Bruckes' reagent, Mayer's reagent, cadmium iodide, tannic acid. None of these gave a precipitate with diamino acids in acid solution. Tannic acid only precipitates in neutral solution. This precipitate was examined and found to give the same result as the phosphotungstic acid precipitate.

The above work was carried out under the direction of Professor S. B. Schryver.

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LIX. THE HEMICELLULOSES. I. THE HEMICELLULOSE OF WHEAT FLOUR.

By DONALD HERBERT FRANK CLAYSON
and SAMUEL BARNETT SCHRYVER.

From the Biochemical Department, Imperial College of Science and Technology.

(*Received June 6th, 1923.*)

Some years ago, Schulze described the preparation of nitrogen-free residues from leguminous seeds, which yielded on hydrolysis with dilute acids reducing sugars. The precursors of these sugars which were not actually isolated were called by Schulze "hemicelluloses[1]."

During the course of some researches on the constituents of the cell-wall substances of plants by Clayson, Norris and Schryver [1921], it was found that, by means of *N* sodium hydroxide solution, substances could be extracted from all the materials investigated, which were soluble in the more concentrated alkaline solutions, but not in very weak alkali; they could be precipitated from the alkaline solutions on addition of acids, and as they yielded reducing sugars on hydrolysis with acids, they were assumed to belong to the class of products termed by Schulze the "hemicelluloses." Most of the products isolated gave on hydrolysis relatively large amounts of pentoses, and all of them gave a blue coloration with iodine.

It was decided to continue investigations with the object of isolating in bulk these so-called "hemicelluloses" and of determining their distribution and later on their function in plant-tissues. The distribution appears to be a very wide one. In some lignified tissues they exist in relatively large quantities ("wood-gums"), and the product isolated many years ago from barley by O'Sullivan and called by him "amylan" also apparently belongs to this class.

The first investigations were carried out on leguminous seeds from which a "hemicellulose" and also "cytopectic acid" [see Clayson, Norris and Schryver, 1921] were isolated and in extending the work to cereals wheat flour was chosen as the chief material for research. By means of the process described in detail below, a product giving a blue coloration with iodine like starch, but differing in many essential properties from this substance, was isolated. It is shown in the second communication [Schryver and Thomas, 1923] that the same product can also be isolated from the commercial starches.

[1] A fuller account of the literature of the hemicelluloses is given in the third paper of this series by Miss O'Dwyer [1923].

Experimental.

Extraction of gliadin. Flour in quantities of 1 kilo. at a time was heated in tin-lined copper vessels, provided with a reflux condenser, with 8 litres of 70 % alcohol. An efficient stirrer, mechanically driven, was attached to the apparatus and the whole mass kept stirred whilst the mixture of flour and alcohol was heated in a water-bath. After about two hours the alcohol was filtered off from the flour on a Buchner funnel, and the flour was returned to the extraction apparatus and treated a second time with 70 % alcohol. By this means nearly all the gliadin was extracted from the flour. This, after the second filtration from the alcohol, was air-dried.

Extraction of the starch. The dried gliadin-free flour was then ground up to a thick paste with water by a motor-driven pestle and mortar and the paste was then thrown into nearly boiling water. About 9 litres of water altogether were used for each kilo. of flour. The mixture of flour and water (which was made in enamelled buckets) was then kept for 1 hour at about 85° by immersing the containing vessel in a bath of boiling water and was stirred from time to time. In this way the starch was gelatinised. The mass was then cooled to 40° and 1 g. of taka-diastase, dissolved in about 500 cc. of water at 40° was added gradually with constant stirring. Toluene and chloroform were then added and the mixture was kept for about 20 hours in an incubator at 37°. After this period, the mixture had lost its gelatinous consistency, and was filtered through coarse muslin, with hand pressure, to separate the undigested lumps. These were afterwards ground up with water in a motor-driven pestle and mortar, and the paste thus obtained either added to the main bulk of the filtrate or to the second digestion mixture obtained by the method about to be described. The filtrate was then passed two or three times through a Sharples centrifuge, using first a wide, and afterwards a finer nozzle, the centrifuging process being repeated (generally three times) until the liquid was nearly clear. The solid matter was then obtained in the form of a thick paste. This was then ground up with water, and, as before, the paste was thrown into nearly boiling water, 9 litres again being used. The mixture was kept at about 80° for 1 hour and again digested with taka-diastase. After the second digestion, the mass usually passed through coarse muslin without pressure and contained very few lumps. The solid was again separated from the liquid by the Sharples centrifuge, and gelatinised and redigested with taka-diastase as before, for a third time. In the third digestion, only about half the bulk of water used in the first digestions was employed. In the earlier experiments the course of digestion was followed out by estimations of sugar. It was found that by far the greater part of the starch was digested on the first treatment with taka-diastase. Practically no sugar was obtained as the result of the third digestion, but this was always carried out as a precautionary measure. The starch-free product was obtained finally in the form of a thick paste.

Extraction of the glutenin. The glutenin is soluble in 0·1 % sodium hydroxide

solution, which is too weak to extract the hemicellulose. In some experiments, the starch-free product obtained as above was dried in a current of air before the extraction of the glutenin. It was then obtained in the form of a very hard horny mass, from which the glutenin is extracted only with very great difficulty. It is therefore advisable to extract the glutenin from the starch-free paste without drying previously. This is accomplished by repeated extractions with weak alkali, the solid being afterwards separated from the liquid by means of the Sharples centrifuge. The extraction should be continued until the liquid no longer gives the biuret reaction. It is difficult to get out the last traces of nitrogen, but these can be eliminated by methods of purification described below.

Extraction of the hemicellulose. The mass remaining after extraction of the glutenin is then treated with cold 4 % solution of sodium hydroxide. An appreciable amount of yellowish amorphous substance remains undissolved. This contains a small amount of cytopectic acid, which could be extracted from it by 0·5 % ammonium oxalate solution by the method previously described [Clayson, Norris and Schryver, 1921]. The remainder consisted of cellulose, which dissolved completely in zinc-chloride-hydrochloric acid solution. The insoluble mass was separated from the liquid by filtration through a thick pad of paper pulp (made up with 4 % caustic soda solution). The filtrate was slightly opalescent. On acidification with acetic acid, it became at first very turbid, but on standing for a short time a very bulky white precipitate separated, leaving a perfectly clear white supernatant fluid. This was filtered off on to a large filter, thoroughly washed with water, graded strengths of alcohol, and then with ether and finally air-dried. It was thus obtained in the form of a light white powder.

Properties of the hemicellulose. When prepared in the manner just described, the flour hemicellulose dissolves fairly readily in boiling water, giving a perfectly limpid solution, from which the hemicellulose very readily separates again on cooling. The solution can be filtered through paper in a hot water funnel. The hemicellulose on drying at 100° loses about 50 % of its weight, and the apparently dry powder holds large quantities, therefore, of the liquids which have been used in its preparation. After drying at 100° it is obtained in the form of a hard granular powder, or sometimes of a horny mass. When in this condition, it does not dissolve readily in water boiling under atmospheric pressure. It readily dissolves, however, when heated with water in an autoclave to 120°. From this solution it readily separates in a non-crystalline form on cooling. It gives a blue coloration with iodine just as ordinary starch does, but resists digestion with taka-diastase. It does not reduce Fehling's solution; when boiled with 1 % sulphuric acid it undergoes hydrolysis, yielding glucose (see next paper). This hydrolysis takes place rapidly and is nearly complete after 2 or 3 hours. As usually prepared, it contains small quantities of nitrogen. It can be purified either by allowing it to separate from its filtered solution in hot water, or by means of the copper salt, a method which is described in detail in the following paper. By these methods it can

be obtained quite free from nitrogen and ash, and then shows a rotation of $[\alpha]_D = +150°$. It is in all respects identical with the hemicellulose obtained from starches, which are described in the paper just quoted.

Origin of the hemicellulose. The hemicellulose was first prepared from flours, purchased in shops, of which the origin was quite unknown. Very varying yields were obtained from different flours. It was of interest to ascertain what the origin of this "hemicellulose" was. It was conceivable that it was an intermediate product derived by the digestion of ordinary starch by taka-diastase. On the other hand, it might be a distinct entity derived either from the starch grains, or from a different part of the plant cell (possibly the cell-wall). To test these points, an estimation of the cellulose was made from two flours of known origin. These were kindly supplied by Dr A. E. Humpharies. They were both obtained from the same mixture of Australian, English and Manitoba wheat. One flour was, however, a "straight-run" flour corresponding to a yield of 73 % of the wheat, whereas the other was more refined and corresponded only to a 23 % yield. It was found that the former gave a yield of 5·4 % hemicellulose (calculated as dried at 100° and protein-free) whereas the latter gave a yield of only 3·6 %. These experiments were each carried out with 1 kilo. of flour. The hemicellulose obtained from each sample (by the methods described above) was not completely purified, but the nitrogen and dry weight were estimated in each. These preliminary experiments on which not too much weight can be put, indicate that the hemicellulose is not derived from the endosperm.

It is possible that the hemicellulose in flour may exert a considerable influence on its value for bread-making and it is proposed to investigate this question in detail.

The authors wish to express their thanks to Dr Humpharies for his assistance in providing materials and one of them is indebted to the department of Scientific and Industrial Research for a grant which enabled him to carry out this work.

Summary.

From wheat flour can be isolated a substance which resists the action of taka-diastase, and is soluble in hot water, in which it gives a perfectly limpid solution from which it separates readily on cooling in an amorphous form. It is soluble in *N* NaOH, from which solution it can be precipitated by acids. It can be purified either by separation from its solution in hot water, or by means of its copper salt, and when thus obtained, nitrogen- and ash-free, has a rotation in *N*/2 NaOH of $[\alpha]_D^{20} = +150°$.

(*Note.* A fuller description of some of the properties is given in the following paper.)

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LX. THE HEMICELLULOSES. II. THE HEMICELLULOSE CONTENT OF STARCHES.

By SAMUEL BARNETT SCHRYVER
and ETHEL MARY THOMAS.

From the Biochemical Department, Imperial College of Science and Technology.

(*Received June 6th, 1923.*)

In the first paper [Clayson and Schryver, 1923] a "hemicellulose" very similar to starch but differing from this in many particulars has been described. Some evidence has been given to show this is not an intermediate product of degradation of ordinary starch by taka-diastase, but a distinct chemical entity. If this were the case, it might be expected that it would be yielded in different amounts by the digestion of ordinary starches and that there might exist some correlation between the amount obtained and the properties of the various starches investigated. This has been found to be the case, and yields varying between practically 0 and 4 % have been found in the six commercial varieties of starch which have been studied.

Preparation of the hemicellulose from starches.

The six starches investigated were those from sago, maize, wheat, rice, tapioca and potato. The authors are indebted to Messrs Hibbert and Co. of Chorley, the Corn Products Co., Manchester, and Young and Strang of Glasgow for their kindness in providing materials for the research.

The method of preparation of the hemicellulose was practically identical with that employed for obtaining it from gliadin-free wheat flour as described in the first paper. The material was gelatinised and treated three times with taka-diastase, the filtrations after each digestion being carried out by means of the Sharples centrifuge in exactly the manner therein described. The residue after digestion and treatment with 0·1 % caustic soda was dissolved in 2–4 % caustic soda solution, from which the hemicellulose was precipitated by acetic acid.

With potato starch, which gives a nearly clear gel on gelatinisation, only an insignificant residue was obtained after two digestions with taka-diastase, whereas sago and maize starches, which yielded on gelatinisation thick pastes,

gave relatively large yields. In each case the digestion was carried out with 1 kilo. of starch and the following yields were obtained:

Origin of starch	Crude hemicellulose %	Nitrogen content %	Nett yield of hemicellulose
Sago	4·1	1·3	3·8
Maize	3·9	1·35	3·6
Tapioca	2·0	1·7	1·7
Wheat	1·65	2·1	1·35
Rice	1·2	2·3	1·0
Potato	Practically none	—	—

The hemicellulose still contained nitrogen (column 3), and if this be in the form of protein, the nett yields of hemicellulose from the various starches may be calculated as given in the last column.

Purification of the crude hemicellulose.

Washing the crude hemicellulose with 0·1 % caustic soda solution was not found to be completely effective in removing the protein. Separation of the hemicellulose from a filtered solution in hot water was somewhat difficult, owing to the ease with which deposition took place, even when a hot-water funnel was used. A method similar to that employed by Baker and Pope [1900] was used. The hemicellulose was dissolved in 2 % NaOH solution and copper sulphate was added till no further precipitation took place. The light blue precipitate was centrifuged off, and washed repeatedly on the centrifuge with 0·2 % NaOH solution. It was then treated with dilute acetic acid and the product thus obtained was washed until the blue copper coloration was removed. It was then treated with graded strengths of alcohol, and then ether and finally air-dried. The white flocculent mass thus obtained still held large amounts of the liquids used in the preparation, but could be readily dried to constant weight by heating to 110°, when it was obtained in the form of a granular powder, free from nitrogen and ash. The specific rotations of the samples in *N*/2 NaOH obtained from the different varieties of starch were practically identical, viz.:

Sago	+151·6°
Maize	+150·6
Wheat starch	+150·0
Tapioca	+151·0
Rice	+150·9
Wheat flour	+150·6

The product gave the following results on combustion:

I. C. 41·3 %; H 6·8 %. II. C 41·6 %; H 6·7 %.

This corresponds with a formula $C_{18}H_{34}O_{17}$ or $(3C_6H_{10}O_5 + 2H_2O)$ (this latter is intended only as an empirical representation of the formula) which requires C = 41·3 % and H = 6·5 %.

Hydrolysis of the hemicellulose.

The hemicellulose is readily hydrolysed by dilute acids. About 20 g. of material were purified by the copper salt method, and the purified product,

without drying, was put immediately into a flask containing about 300 cc. of 1 % sulphuric acid and heated with a reflux condenser for about 6 hours. During the first hour, the greater part of the product disappeared, but the solution remained turbid even after quite prolonged heating. In this and every other hydrolysis carried out, there was always obtained a small amount of a white flocculent substance, which obstinately resisted hydrolysis. The nature of this material, which is only formed in small quantity has not yet been ascertained. After its removal by centrifuging, the clear liquid was treated with the exact requisite amount of barium hydroxide, the barium sulphate was filtered off, and the filtrate concentrated to a syrup under diminished pressure. The syrup on treatment with absolute alcohol gave an almost white solid. From a solution of this an osazone was obtained in large yield, which under the microscope appeared to consist entirely of glucosazone, uncontaminated by the osazone of any other sugar. After recrystallisation it gave a M.P. of 204°. A diphenylhydrazone was also prepared, which agreed in properties with the diphenylhydrazone of dextrose. All attempts to discover other sugars (Seliwanoff's reaction for fructose, Tollens' pentose reaction, etc.) yielded negative results. The solution did not, however, contain pure glucose, for quantitative estimations by Bertrand's method and rotation determinations carried out on the same solution gave instead of a specific rotation of about + 52°, one of about 30°. The glucose was, therefore, contaminated with a substance of lower or opposite rotation.

In other samples of the solution an effort was made to estimate the glucose, by determining the total solids and their reducing power by Bertrand's method. The results of two experiments were as follows:

	I	II
Total solids present	0·1130	0·1445
Glucose therein by Bertrand's method	0·1200	0·1469

The reducing power is therefore a little larger than it should have been if the total solids had consisted entirely of glucose.

Finally, an effort was made to obtain the glucose pure by a method employed by Monier-Williams [1921]. The syrup obtained by the hydrolysis of hemicellulose was taken up in pure methyl alcohol. A small amount of amorphous matter remained undissolved. The filtrate from this was evaporated to a syrup which was again taken up with methyl alcohol, the solution was boiled with animal charcoal, filtered, and evaporated to a syrup. This on standing in a desiccator commenced to crystallise. A small portion of it was dried at 100° and the rotation of the solid residue was determined. This was found to be about + 40°. The crystals which separated out had an $[\alpha]_D^{20}$ of + 52·4°. There is no doubt, therefore, that the main product of hydrolysis is glucose. This is, however, contaminated with a small amount of other substances including one insoluble in water. Probably they are of a dextrin-like character, such as are generally obtained on the hydrolysis of starch by dilute acids. On hydrolysing the hemicellulose with 1 % oxalic acid at 120° an insoluble product was also obtained.

SUMMARY.

A method is described for isolating from the starches a hemicellulose identical with that previously described as having been obtained from wheat flour. The substance has an $[\alpha]_D$ in $N/2$ sodium hydroxide solution of $+ 150°$. It has a composition corresponding with $C_{18}H_{34}O_{17}$ ($3C_6H_{10}O_5 + 2H_2O$). It yields on hydrolysis with dilute acids glucose as the only reducing sugar mixed with small amounts of other products, probably of a dextrin-like character. The various starches give yields of this substance varying from practically 0 in the case of potato starch up to nearly 4 % in the case of sago.

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LXI. THE HEMICELLULOSES. III. THE HEMICELLULOSE OF AMERICAN WHITE OAK.

By MARGARET HELENA O'DWYER.

From the Biochemical Department of the Imperial College of Science and Technology.

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Introduction. The term "hemicellulose" was employed by E. Schulze [1892] and B. Schulze [1894] to denote a substance somewhat similar in character to cellulose, which is easily hydrolysed by weak acids.

E. Schulze [1892] isolated this substance from leguminous seeds by treatment with dilute alkali, and then precipitating the filtrate obtained with dilute acid. He also obtained it from wheat bran and rye bran by the same method. On hydrolysing the product obtained from all three sources, he found that it yielded, in each instance, arabinose and xylose.

Schulze and Tollens [1892] obtained xylose from wheat straw and maize stalks by the same method. From beech wood sawdust Wheeler and Tollens [1889] obtained xylose, and Allen and Tollens [1890] found the same sugar in cherry wood.

Ulander and Tollens [1906] obtained from the hemicellulose of lichens on hydrolysis, glucose, mannose and galactose; while Tottingham, Roberts and Lepkowsky [1920] found xylose, glucose and small amounts of galactose in the alcohol-soluble fraction of the products from the acid hydrolysis of the hemicellulose of apple wood.

Castoro [1906] found that hemicellulose from the seeds of *Ruscus aculeatus* yielded mannose and a small quanitity of arabinose on hydrolysis. From the husks of the same seed he obtained galactose.

Again Castoro [1907] found that the husks of *Cucurbita Pepo* seeds contained xylan, as xylose crystals were isolated from the syrup obtained after the husks had been hydrolysed with sulphuric acid, and that a galactan was also present, as the mother liquor from the xylose crystals yielded mucic acid when oxidised.

E. Schulze and Pfenninger [1910] obtained some interesting results from the pods of *Pisum sativum* and of *Phaseolus vulgaris*. Both of these were found to be rich in hemicellulose, the amount increasing as maturation advanced. Thus the unripe pods of *Pisum* gave 16–19 %, whilst the ripe pods yielded 48·6 %. On hydrolysis, galactose and arabinose were obtained, but the hemicellulose was not actually isolated.

Although isolated instances of this kind occur throughout the literature,

no systematic attempt seems to have been made, so far, to examine the "hemicelluloses" obtained from various sources, and to compare the results obtained in each case. Most of the estimations seem to have been carried out on seeds.

Before the work described in this paper was begun experiments carried out in this laboratory by Miss E. M. Thomas [Schryver, 1922] on small amounts of American White Oak sawdust, showed that alkali extracted substances in amounts varying greatly in different specimens of timber. These substances appeared to be somewhat similar to those obtained by E. Schulze [1892], and called by him the "hemicelluloses."

It was then decided to endeavour to isolate the product, or products, from large quantities of the American White Oak, and to investigate its properties and the products of hydrolysis. The sawdust employed was obtained directly from the cooperage of the brewery of Messrs Whitbread and Co., through the courtesy of Mr H. C. Sweetman. No further information as to its origin was available beyond the fact that it was from American White Oak. It is hoped later to continue the investigation of the hemicelluloses from timbers, the origins of which are authenticated, with the object of determining the distribution and functions of these substances.

Method of Preparation. In the first instance, 600 g. of the sawdust were extracted several times with hot water and dried. 6 litres of 4 % caustic soda were then added to the dried material and the mixture was left standing for two days, during which time it was well stirred with a mechanical stirrer. It was next filtered through fine muslin on a Buchner funnel, the sawdust being well washed with weak alkali. A slight excess of acetic acid was added to the filtrate and washings, and a greyish-white precipitate of a sticky character which was found to be partly re-distributed in water, was formed. The filtrate appeared to be of a colloidal nature and precipitation was not complete until an equal volume of 95 % alcohol had been added. The precipitate brought down by the alcohol was a flocculent one. The whole of the precipitate was then collected on a filter, washed with 50 % alcohol, and purified by re-dissolving in 4 % caustic soda, filtering through paper pulp, and re-precipitating with glacial acetic acid, and 95 % alcohol. After washing with various grades of alcohol and finally with ether, the resulting product was dried first in the air, and then in the oven at 105° and weighed. It was found that 62·45 g. of the products were obtained from 600 g. of sawdust, a yield of a little over 10 %.

50 g. of this material were now further purified by a method described by Baker and Pope [1900]. It was first extracted with 2 % caustic soda on the water-bath, and filtered through glass wool. An excess of Fehling's solution was then added, and the voluminous blue precipitate was washed several times with very dilute alkali. Cold dilute acetic acid was then added to decompose the copper compound, and the mixture washed first with very dilute acid and then with dilute alcohol. The precipitate was next treated with boiling absolute alcohol and washed with cold 95 % alcohol to get rid of the

last traces of acid. After washing with alcohol and then with ether, it was allowed to dry, first in the air, and then in the oven at 105°.

Properties. The product obtained by this method consists of a fine greyish-white amorphous mass, free from nitrogen and from inorganic matter. It is soluble in boiling water, forming a thin gelatinous mass on cooling. It does not dialyse through parchment to any extent. On addition of iodine to the solution, a slightly greenish-blue coloration is given. It does not reduce Fehling's solution and is laevo-rotatory. After purification of the product by the copper method as described above, the rotation $[\alpha]_D = -75°$, in 1 % caustic soda solution, was found. The following figures were obtained by combustion:

(1) 0·1874 g.; 0·3070 CO_2; 0·1089 H_2O; C = 44·67 %, H = 6·40 %.

(2) 0·1974 g.; 0·3239 CO_2; 0·1130 H_2O; C = 44·74 %, H = 6·35 %.

The examination of the hydrolysis products shows that the substance is probably a mixture of nearly 70 % xylan + araban, and 30 % mannan + galactan, a very small percentage of material remaining undissolved after hydrolysis. A mixture of 70 % araban + xylan ($C_5H_8O_4$) and 30 % mannan + galactan ($C_6H_{10}O_5$) requires C = 45·14 % and H = 6·12 %.

On hydrolysing 0·4 g. of the product with 12 % hydrochloric acid and treating the distillate with phloroglucinol, the amount of furfural phloroglucide precipitated was found to be 0·2730 g., which, reckoned as pentose, gives a yield of 70 %. On treating the contents of the crucible containing the furfural phloroglucide with alcohol, according to the method described by Tollens and Ellett [1905], for the identification of methylpentose, no loss in weight occurred, and the alcoholic filtrate showed no change in colour. It is indicated therefore that there is no methylpentose present.

In order to ascertain the relation between the time of hydrolysis of the product with dilute sulphuric acid and the amount of reducing sugars present, 0·5 g. of the product was subjected to hydrolysis with 1 % sulphuric acid for periods of two, three, four and six hours. Bertrand's method was employed to ascertain the reducing power of the hydrolysed solution in each case.

It was found that:

After	2	hours'	hydrolysis	43·74 %
„	3	„	„	51·24 „
„	4	„	„	59·0 „
„	6	„	„	61·74 „

of reducing sugar, reckoned as glucose was obtained.

The isolation of the hydrolysis products.

Preliminary Experiments. These preliminary experiments were carried out with a product which had not been purified by the copper method.

20 g. of the product were hydrolysed with 200 cc. of 1 % sulphuric acid for six hours. The hydrolysed solution at the end of this time still contained a small proportion of insoluble matter. The solution was filtered and a suffi-

cient quantity of barium hydroxide added exactly to neutralise the solution, which was then filtered and concentrated to small bulk under reduced pressure. The solution was now in a syrupy condition. Some 95 % alcohol was added to get rid of any barium sulphate remaining, and after filtering, the solution was again concentrated as before. A little more alcohol was then added and the syrup was allowed to evaporate slowly over sulphuric acid. The crystalline mass which slowly formed was then heated for a long time on the water-bath with 95 % alcohol [E. Schulze, 1892] and left to stand over concentrated sulphuric acid. After some time a white crystalline mass formed. The mother liquor was then filtered off and to 20 cc. of this, 2 g. phenylhydrazine hydrochloride and 3 g. of sodium acetate were added. The mixture was heated in a test-tube in a boiling water-bath for ten minutes and at the end of that time an orange-yellow osazone separated out. This was filtered off, and on again heating the mixture in the water-bath for some time indications of a second osazone appeared, with the formation of some brownish oily drops.

The first formed osazone was now washed with water and dried and gave a melting point of 156°. On recrystallisation from 60 % alcohol the constant melting point was found to be 160°. It appeared to be the osazone of xylose. As a means of further identification Bertrand's reaction [1891] was now carried out on another portion of the solution from which the osazone was formed. To 2 g. of the sugar syrup, 2·5 g. of bromine and 5 g. of cadmium carbonate were added. The mixture was then gently warmed in a test-tube and after corking loosely set aside for twenty-four hours. At the end of that time the solution was evaporated almost to dryness, taken up with water and again evaporated almost to dryness. On the addition of a little alcohol a crystalline mass formed which at first appeared somewhat amorphous under the microscope, but on recrystallisation from alcohol, the characteristic boat-shaped needles of the double cadmium salt of xylose $(C_5H_9O_6)_2\,Cd + Cd\,Br_2 + 2H_2O$ were deposited. A third portion of the same sugar syrup was boiled with some paraformaldehyde and after a time a white mass of crystals separated out. These were found to have a melting point of 55°. *l*-Xylose diformal melts at 56° and has the formula $C_5H_6O_5\,(CH_2)_2$ [Lobry de Bruyn and Van Eckenstein, 1903]. The osazone of *l*-xylose, the double cadmium salt of the same sugar, and *l*-xylose diformal have therefore been obtained from different portions of the same sugar syrup.

The mass of crystals which had been filtered off from the mother liquor were now dried and examined. 1 g. was treated as before with 2 g. of phenylhydrazine hydrochloride, 3 g. of sodium acetate and 20 cc. of water [Mulliken, 1905] and after heating in a test-tube in a boiling water-bath for a very short time some colourless crystals separated out. After more than fifteen minutes an orange-yellow osazone formed and brownish-yellow oily drops appeared at the surface. After filtering off the osazone, washing as before, and recrystallising from 60 % alcohol, it was found to have a melting point of 160°. As the osazones of both xylose and arabinose give this melting point, further

means of identification had to be sought. An attempt was therefore made to obtain the diphenylhydrazone of arabinose by treating the syrupy solution of the sugar with slight excess of diphenylhydrazine [Neuberg, 1900, 1904; Browne, 1912]. Sufficient alcohol was added to form a perfectly clear solution and the mixture was boiled for half an hour in a water-bath in a flask connected with a reflux condenser. After standing for some time a white crystalline mass separated out from the solution. The crystals were washed with a very little cold alcohol and dried in the water oven. They gave a M.P. of 196°. After recrystallisation a M.P. of 199° was obtained with rapid heating. Tollens and Maurenbrecher [1905] observed a M.P. of 204°–5° for the diphenylhydrazone of arabinose, but Haar [1920] points out that it is difficult to obtain a constant M.P. higher than 200°.

Second Hydrolysis. 1200 g. of sawdust were now subjected to the same treatment as before, and yielded 121·05 g. of the product; 60 g. of which were hydrolysed with 300 cc. of 1 % sulphuric acid. The hydrolysed solution was again neutralised with barium hydroxide and concentrated as before. Four volumes of 95 % alcohol were then added and the solution, after filtering, was concentrated a second time to very small bulk and decolorised with norite. After further addition of alcohol and a third concentration, the resulting syrup was divided up into two portions. Absolute alcohol was added to the first portion and a large amount of precipitate formed. This, after drying, was treated with hot 80 % alcohol and filtered. As mannose is soluble in hot 80 % alcohol it was hoped to obtain the greater part of this sugar, if present, in the filtrate. This filtrate was again divided into two portions, one of which was left standing over concentrated sulphuric acid. To part (*a*) of the remainder, one volume of phenylhydrazine and one volume of 50 % acetic acid were added. After the mixture had stood for a short time, colourless rhombic crystals were formed. These were left in the solution over the week-end and had disappeared on the following Monday morning. On warming the solution for a short time a mass of sheaf-like crystals, entirely different from those first formed, appeared. They closely resembled crystals of glucosazone under the microscope. After recrystallisation from 60 % alcohol they gave a constant melting point of 204°. This melting point is that of the osazone of mannose.

Part (*b*), the remainder of the second portion of the last filtrate, was treated with phenylhydrazine and 50 % acetic acid as before. After standing for a short time the rhombic crystals again appeared. Some colourless needles were also seen under the microscope. The crystal mass was collected after twenty-four hours, dried and recrystallised from hot water. The melting point before recrystallisation was found to be 178° with rapid heating; after repeated recrystallisations from hot water a fairly pure hydrazone was obtained, melting at 196°. The low melting point obtained in the first instance is probably due to the fact that some crystals of the hydrazone of galactose were present. $[\alpha]_D$ (in pyridine) = + 22°. Haar [1920] gives a value for mannose phenylhydrazone $[\alpha]_D$ = + 26·61°.

That portion of the solution obtained with hot 80 % alcohol which had been left standing over sulphuric acid, was now examined. Part of it was found to have separated out as a hard white amorphous mass. On drying this and treating with 90 % alcohol under reduced pressure crystals were formed after the syrup had stood a short time. These were dried on a suction filter and washed with small amounts of alcohol and ether, and then recrystallised from alcohol. They gave a constant melting point of 136°. $[\alpha]_D$ (in 10 % aqueous solution) $= + 15{\cdot}3°$. On adding phloroglucinol to the sugar solution no coloration was given. The crystals are evidently mostly those of mannose, with probably a little galactose also present. The rest of the syrup obtained from the second hydrolysis with 1 % sulphuric acid was used later on for a rough qualitative estimation of two of the sugars which had previously been identified.

Third Hydrolysis. 20 g. of the purified product were hydrolysed as before. There was still present a small insoluble residue which after drying and weighing, was found only to amount to 0·45 %. The hydrolysed solution was neutralised as before with barium hydroxide, treated with alcohol and evaporated to a syrup. After concentrating and taking up with alcohol three times an attempt was made to separate the sugars, which previous experiments had shown to be present, by their differences in solubility in alcohol and in water. The results have been fairly satisfactory and it is hoped that further work along these lines may be even more successful. The sugar syrup was decolorised with norite, and, after concentration the third time to very small bulk under reduced pressure, was dried over sulphuric acid, precipitated with hot absolute alcohol and filtered immediately. As xylose is soluble in hot absolute alcohol to a much greater extent than mannose, arabinose or galactose, it was hoped to obtain this sugar in a fairly pure condition in the filtrate. The filtrate was again concentrated under reduced pressure and left to crystallise out over sulphuric acid. After some time crystal aggregates consisting for the most part of fine needles appeared. These were sucked dry on the filter, washed with a little alcohol and ether and then recrystallised from alcohol. They gave a melting point of 156°. $[\alpha]_D$ (in 10 % aqueous solution) $= + 20°$. On heating a solution of the sugar with phloroglucinol, a greenish-black precipitate appeared. According to Mulliken [1905] the melting point of xylose is 150–153° and its specific rotation + 18·7°.

The precipitate obtained by adding hot absolute alcohol was left dry over sulphuric acid and then just sufficient water was added to dissolve up the crystals. After concentrating to a slight extent under reduced pressure, the solution was left over sulphuric acid. Crystals began to separate out from the aqueous solution. These were collected and dried on the suction filter, the mother liquor being again treated with absolute alcohol and left over sulphuric acid. The crystals, on drying, gave a melting point of 116°. On recrystallisation from absolute alcohol a mass of fine hexagonal crystals formed. These melted at 167°. A solution of the crystals gave a very slight coloration with phloro-

glucinol. Galactose [Mulliken, 1905] melts with rapid heating at 168° and has a specific rotation of + 80·3°. A value for $[\alpha]_D$ of + 82° was obtained in 5 % aqueous solution. Owing to the small amount of the sugar present, the mucic acid estimation [Fernau, 1909] could not be carried out at this stage, but it was done later on a solution containing a mixture of the sugars. The phenylhydrazone was obtained by dissolving some of the sugar crystals in a little water and adding phenylhydrazine and 50 % acetic acid as before. After the mixture had stood for about two hours the hydrazone separated out in the form of fine colourless needles, similar to those seen when the hydrazone of mannose was prepared. These were washed with ether and then with a little alcohol and ether. After drying, these were found to have a melting point of 156°. The specific rotation was not obtained. The mother liquor from the last filtration which, it was thought, would probably contain arabinose, some mannose, and perhaps a little galactose was concentrated under reduced pressure and treated several times with hot absolute alcohol. Mannose is slightly soluble in hot absolute alcohol, and galactose is a little less insoluble in the same reagent than arabinose, hence after repeated extractions with hot absolute alcohol, it was hoped to obtain a fairly pure specimen of arabinose. The precipitate which remained after these extractions was dried on the suction filter after standing over sulphuric acid and recrystallised from hot absolute alcohol. The crystals were filtered off, washed with a little alcohol and ether and dried over sulphuric acid. They gave a constant melting point of 160°. $[\alpha]_D$ (in 10 % aqueous solution) = + 103°. On treatment of the sugar solution with phloroglucinol a purplish-black precipitate appeared.

The sugar is therefore identified as *l*-arabinose.

In order to obtain an approximate idea of the amounts of xylose and arabinose present, the diphenylhydrazone of arabinose was obtained by the method described in a previous part of this paper. The mixed sugar syrup from the second hydrolysis, after being treated with alcohol, and concentrated under reduced pressure was allowed to crystallise out over sulphuric acid. 2 g. of the mixed sugar crystals were then dissolved in a little water and 2·5 g. diphenylhydrazine added. When the hydrazone had separated out, the crystals were filtered into a weighed Gooch crucible, washed with a very little alcohol, dried in the water oven, and weighed. It was found that 0·780 g. of diphenylhydrazone $C_{17}H_{20}O_4N_2$ was obtained from 2 g. of the mixed sugars. In order to obtain the amount of arabinose ($C_5H_{10}O_5$) which this figure represents, it was multiplied by 150/316 = 0·4747.

$$0{\cdot}780 \times 0{\cdot}4747 = 0{\cdot}3703 \text{ g. arabinose} = 18{\cdot}5 \text{ \% (approx.)}.$$

As the total amount of pentose present was found to be 70 % the percentage of xylose may be roughly calculated as 51·5 %.

An estimation of the amount of mucic acid formed from the mixed sugars was now attempted in order to obtain some idea of the relative amount of galactose, and hence of mannose present. A modification of the Tollens method

described by Fernau [1909] was employed for this purpose. It was found that 4·89 g. of mixed sugar crystals gave 0·3640 g. of mucic acid, or

$$0{\cdot}3640 \times 1{\cdot}33 = 0{\cdot}4841 \text{ g. galactose.}$$

If the sugar from which the mucic acid was obtained were pure galactose, this would represent 70 % of the galactose present, according to Fernau. The percentage of the sugar present would, therefore, be 6·9, approximately. In a mixture of sugars, however, the percentage of galactose obtained by the mucic acid estimation would probably be much lower, and besides it is not likely that mannose is present in any great amount, as it is found mostly in gymnosperms [Schorger, 1917]. Pinoff's test [1905] for fructose, gave a negative result.

It was not found possible to form either the hydrazone or the osazone of glucose from the mixed sugar crystals. On treating the crystals with diphenylhydrazine only the diphenylhydrazone of arabinose was formed. It is therefore assumed that glucose is not present.

Summary.

Apparently the chief product of hydrolysis of the hemicellulose of American White Oak is *l*-xylose. According to Browne [1912, p. 553], xylan, with a little araban, makes up about 15–25 % of the dry matter of deciduous trees. Arabinose is apparently present in much smaller quantities than xylose in American White Oak. A rough quantitative estimation gave the amount of xylose yielded by the hemicellulose of this wood as 51·5 % and that of arabinose as 18·5 %.

Hexoses apparently form about 30 % of the products of hydrolysis. These have been identified as mannose and galactose. A mucic acid estimation of the galactose gave approximately 7 %, but as the estimation was made on the mixed sugars, the true figure is probably very much higher, as the amount of mannose present appears to be very small.

It is impossible to say in what combination these products exist in the wood. Probably in the form of an arabo-xylan and a manno-galactan.

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LXII. PECTIN AND ITS HYPOTHETICAL PRECURSOR, "PROTOPECTIN."

By FRANK TUTIN.

From the University of Bristol Agricultural and Horticultural Research Station, Long Ashton.

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It has long been known that the expressed juice of unripe fruits contains very little pectin, and that when the crushed marc is subsequently extracted with cold water, only small, additional quantities of this substance are obtained. In contrast to this, when a soft, fully ripe fruit is pressed, the juice contains a considerable amount of pectin, and further and larger quantities may readily be obtained by extraction of the marc with cold water.

When, however, an unripe fruit is heated with dilute hydrochloric acid it becomes thoroughly disintegrated, and the liquid obtained by straining and pressing the resulting mixture is rich in pectin. This has led to the assumption that unripe fruits contain an insoluble compound which might be regarded as the parent substance of pectin, and which, by hydrolysis, during the process of ripening, or when heated with acid, becomes converted into ordinary, soluble pectin. To this insoluble compound the name "protopectin" was given by Fellenberg [1918], and it corresponds to the "pectose" of earlier investigators. It has, moreover, been assumed that the presence of this "protopectin" was a cause of the hardness of unripe fruits, and that its gradual change into soluble pectin was a feature of the process of ripening.

After having worked for some time with pectin, however, it occurred to the present author that the generally accepted explanation of the apparent deficiency of soluble pectin in unripe fruits might not be the correct one. To quote one observation: if it be attempted to filter a solution containing only a relatively small concentration (0·5 % or even less) of pectin through a reasonably efficient paper it will be found that, after a very few moments, filtration becomes extremely slow. The filtrate then collected contains only a very small proportion of pectin, the greater part of the latter substance being retained on the surface of the filter as a gel-like layer. Considerable quantities of distilled water may then, in course of time, be passed through this filter, when the pectin continues to be removed, but at an extremely slow and apparently constant rate. Now it seemed that conditions analogous to this might prevail in the case of hard, unripe fruit, where really efficient disintegration of the tissue is peculiarly difficult to attain, particularly when it is considered that

fruits contain products which, though soluble in alcohol, are insoluble in water, and would certainly hinder the access of the latter to the pectin occluded in the tissue.

In view of these considerations, and also taking into account the fact that no direct evidence of the existence of "protopectin" has ever been obtained, the following alternative explanation of the fact that fruits not fully ripe retain part of their pectin content in an apparently insoluble form, presented itself to the author: namely, that no such substance as "protopectin" exists, but, that the persistent retention of pectin in an apparently insoluble form by the tissue of incompletely ripe fruits is due partly to the presence of substances insoluble in water but soluble in alcohol, but more particularly to the great difficulty in attaining, by mechanical means, really efficient disintegration of the unripe tissue. In other words, it is suggested that the presence of "protopectin" is not a cause of the hardness of the tissue of unripe fruits, but that the behaviour of this tissue, described above, is the effect of its dense nature.

More than two years ago a few preliminary observations were made which tended to support this view. The material employed was a very finely ground sample of dried pomace (the press-cake from the cider press). Three quantities, each weighing 30 g., were exhaustively extracted in a Soxhlet apparatus; No. 1 with water only; No. 2, first with alcohol, and subsequently with water; whilst No. 3, after extraction with alcohol, was heated in an autoclave at 110° with dilute hydrochloric acid for one hour, and subsequently exhausted with boiling water. The amounts of pectin obtained from the three extracts were, respectively, 1·4 g., 1·6 g., 1·6 g. Had "protopectin" been present the last extract should have yielded the largest amount of pectin, whilst the inhibiting effect of the presence of alcohol-soluble substances is shown by the lesser amount of pectin obtained from the first extract.

It was therefore decided to conduct some experiments with unripe apple tissue, taking precautions to remove all the substances soluble in alcohol and also to ensure as complete a mechanical disintegration as was possible with the means available.

The variety of apple selected for this purpose was Bramley's Seedling. The fruit for the experiment described below was picked on August 29th, 1922, when it was far from being in a ripe condition. These apples, when pressed, yielded a juice which contained only a very small amount of pectin. A quantity of the fruit was peeled, deprived of all core tissue, thinly sliced, and then placed in alcohol, these operations being conducted as quickly as possible. The weight of fresh, sliced material taken was 286 g. The alcohol into which the sliced fruit had been placed was decanted, and replaced by fresh solvent, and this treatment was continued, at the ordinary temperature, so long as anything was dissolved from the apple tissue. The latter was then well crushed in a mortar, brought on to a filter, and well washed, first with absolute alcohol, and finally with ethyl acetate. After drying, first in the air, but subsequently in a vacuum over sulphuric acid, the material weighed only 8·9 g. This dry

material was then submitted to a very thorough grinding in a mortar and the whole of it passed through a sieve the meshes of which were approximately $\frac{1}{200}$th of an inch square. The ground product then weighed 8·8 g. This finely ground tissue was then well stirred, at frequent intervals, for about 2 hours with 1300 cc. of cold distilled water[1], and subsequently allowed to settle for about 22 hours. The supernatant liquid was then decanted, and the process repeated. In all, 25 extractions were made. The pectin content of each of the last ten extracts was very small, and apparently constant. The residual, wet marc was then mixed with about a litre of alcohol, collected on a Buchner filter, well washed, first with alcohol and subsequently with ethyl acetate, pressed, and allowed to dry. The dry material was re-ground with fine, sifted sand[2] in a very thorough manner, so that the sand became crushed to microscopic, sharp-edged, flint-like fragments. After again passing the product through the above-mentioned sieve the extractions with water were repeated as before. In this case it was found that only ten extractions were necessary to reach the point where only a very small amount of pectin was removed. The marc was then washed with alcohol and ethyl acetate, and dried, as previously described, after which it was again very thoroughly ground with the finely comminuted sand which had passed through the sieve after the first re-grinding. On repeating the treatment with water, as before, only seven extractions were found necessary. At this stage the material was carefully examined under a microscope, samples being taken two minutes after it had been vigorously stirred with distilled water, some from near the surface of the mixture, and others from the lowest portion. The former showed only fragments of cell walls together with minute particles of silica, whilst the latter contained, in addition to silica, some small fragments of vascular tissue which has escaped complete disintegration, together with one or two groups of about three partially broken cells still adhering together. It was evident that disintegration had been very thorough, although not absolutely complete, and it was concluded that a small amount of soluble pectin would still be retained in the tissue. Nevertheless, it was not deemed necessary to continue the laborious process of grinding and extracting in order to obtain proof of the absence of "protopectin." The residual marc was therefore heated in an autoclave at 110° with $N/20$ HCl under the conditions described by Carré [1922] and subsequently extracted with distilled water four times, in the manner described. The fourth extract contained an amount of pectin that was scarcely detectable. The pectin present in all the four series of extracts, obtained as above described, was, in each case, directly estimated as such. After concentration under diminished pressure, each extract was treated with a large volume of alcohol, the pectin collected, dried in a vacuum and weighed. The method proposed by Carré and Haynes [1921] was not adopted, as their procedure represents

[1] In order to ensure that the dry powder would readily mix with the water it was found convenient in the first instance to moisten it with a little alcohol.

[2] Purified by extraction with acids followed by strong ignition.

as pectin any acid which might happen to be present and which yields a calcium salt insoluble in dilute acetic acid. The following is a summary of the results obtained by the present author:

Weight of fresh apple tissue 286 g.	Weight of ground and dried tissue after treatment with alcohol 8·80 g.	Volume occupied by dry ground material About 28 cc.
	Weight of pectin from 8·80 g. of dry tissue g.	Volume occupied by wet tissue on subsidence from water (approximate) cc.
1. After fine grinding	1·9347*	330
2. After re-grinding with sand	1·5104*	220
3. Again re-ground with sand	0·3238	110
4. Autoclaved with HCl	0·1005	115

* These two weights, particularly the first one, are very slightly greater than the actual weight of pectin obtained. This is because, during the decantation of the pectin solutions from the marc, a very small amount of the latter was unavoidably removed with the pectin solution, and it was not subsequently separated owing to the above-mentioned difficulty of filtering, through a good paper, solutions of pectin which are not very dilute. The liquids decanted after the third and fourth treatments, however, were each exactly filtered, so that the weights of pectin yielded by them are strictly comparable. Nevertheless, the total percentage of pectin calculated on the weight of fresh tissue taken appears to be high in comparison with results previously obtained. This is attributed, in part, to the efficiency of the grinding and extraction, but also, probably, to the unripe condition of the fruit, since fully ripe fruit from the same trees was found to contain at least 50 % more water.

The volumes occupied by the marc are given as supplying additional evidence of the change produced by the various treatments.

It is seen from the above results that, after the removal of the "soluble pectin" from the original, finely ground tissue, a further, and almost as great an amount may readily be dissolved by treatment with cold, distilled water when the material has merely been submitted to a process of more efficient mechanical disintegration. The amount of pectin subsequently obtained after treatment with acid is insignificant, and its retention by the marc is obviously due to the fact that quite complete disintegration of the tissue had not been attained, even after the drastic grindings that had been resorted to.

The only conclusion that can be arrived at is, therefore, that "protopectin" does not exist, but that all the pectin present in the apple occurs in the ordinary, soluble form. The problem of estimating pectin in fruits therefore appears to resolve itself, in the first instance, into one of attaining complete disintegration of the tissue. It seems likely that this would most readily be accomplished by resorting to the treatment with dilute HCl employed by Carré [1922] for the hydrolysis of the supposed protopectin.

After the commencement of this work a paper, referred to above, was published by Carré, dealing with the changes which occur in the "pectic" constitutents of stored fruit. The conclusions arrived at during the present investigation offer a ready explanation of some of the results there described. In the first place, it is probable that the "considerable variations" in the pectin content of stored fruit observed by Carré were due, not so much to a large sampling error, as suggested by her, but to differences in the degree of mechanical disintegration of the various samples of tissue before extraction

of the "soluble pectin." The great deficiency of pectin found by her in the early stages of ripening would appear to be chiefly due to lack of efficient disintegration, which is especially difficult to attain when the fruit is in an unripe condition. No special precautions seem to have been taken by Carré to ensure thorough grinding, and, in fact, she appears to recognise that this had not been attained. Thus, on p. 708, when describing the estimation, by treatment in an autoclave at 110° with dilute HCl, of protopectin in tissue which was supposed to have been already deprived of soluble pectin, she states: "The material became thoroughly disintegrated during the process...." Then again, she states that "A series of estimations of pectin and protopectins carried out at regular intervals on the same apples, showed that a very definite relationship exists between them and that the changes in the two constituents tend to be equal and opposite in amount." This, also, is obviously another result of varying degrees of inefficient mechanical disintegration. Naturally, the more pectin that can be removed after the first process of partial disintegration, the less there is left to be removed when the product has been submitted to a process which ensures complete disintegration.

It was observed by Carré that after crushing, and thoroughly washing apple tissue with water, a small amount of pectin continued to be removed during several weeks, on further treatment with water. This behaviour is attributed by the present author to the slow rate of diffusion of pectin from insufficiently disintegrated tissue.

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LXIII. MECHANISM OF OXIDATION IN THE PLANT.

PART I. THE OXYGENASE OF BACH AND CHODAT: FUNCTION OF LECITHINS IN RESPIRATION.

By PATRICK HUGH GALLAGHER.

From the Department of Biochemistry, Cambridge.

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In the ordinary guaiacum test for plant oxydases two types of reaction are met with according to the variety of plant examined. Several species, notably *Russula* and *Lactarius*, are capable of reacting directly with a fresh alcoholic solution of guaiacum resin with production of its characteristic blue oxidation product. On the other hand, the majority of plant species only give the guaiacum reaction in presence of hydrogen peroxide, or of such substances as peroxidised turpentine, etc. In an attempt to correlate these two types of reaction Bach and Chodat [1903, 1] suggested the existence of two enzymes in the plant oxidative system: (i) a substance which they termed "oxygenase," which has the property of fixing atmospheric oxygen in such a manner as to produce a peroxide, and (ii) a "peroxydase" which catalyses the decomposition of the peroxide, with liberation of active oxygen. The view of these authors has been rather generally, though not universally, accepted. It is admittedly questionable as to whether all the so-called direct oxydases are resolvable into the two types of constituents suggested. At the same time it will be realised that if plants which give only the indirect, or peroxydase, reaction are, in the ordinary course of metabolism, to effect oxidations at all comparable to that of guaiacum or of the other reagents usually employed, some substitute within the plant for the hydrogen peroxide which must be added in the ordinary laboratory test, will be admittedly essential.

Existence of Peroxides in Plant Juices.

The existence of peroxides in plant juices has given rise to a certain amount of controversy. De Clermont [1875] reported the existence of hydrogen peroxide in vegetable juices. His observation was supported by Griesmayer [1876], Béchamp [1882], and by Bert and Regnard [1882]. Bellucci [1878] could not confirm the observation of these authors by means of the ordinary chromic acid reaction. It is to be observed, however, that the chromic acid reaction, though sensitive for hydrogen peroxide, is rarely given by other peroxides. Generally speaking, it is a matter of no little difficulty to distinguish between hydrogen peroxide and peroxide derivatives when the substance in question is present in relatively small quantity. The reactions, therefore,

which the above authors attributed to hydrogen peroxide may possibly have been given, instead, by organic peroxides present in the cell-sap. Bach and Chodat [1902] showed that the juice of *Lathraea squamaria* contained a substance which turned potassium iodide-starch paper blue. It could be precipitated with 1 % baryta solution. On washing the precipitate and decomposing with weak acid the product was found to be non-reactive towards titanium sulphate, but continued to react with potassium iodide-starch paper. The solution gave no reaction for nitrous acid. They therefore concluded that the reactive substance present was a peroxide. Onslow, M. W. [1919, 1920] also brought forward evidence in favour of the formation of peroxides in various plant extracts.

Evidence obtained in the course of the present work indicates that a substance, or substances, of a peroxide nature is formed in plant extracts in contact with air. It is a matter of common experience that the outer portion of vegetable roots is found to give the direct oxydase reaction with guaiacum, while the inner portion of the root only gives the indirect or peroxydase reaction. If, for instance, a thin section be cut from the surface of a potato and the portion of the tuber so exposed be treated with fresh guaiacum tincture, an immediate intense blue coloration is obtained. If a tuber be cut in two through the centre, a solution of guaiacum poured over the fresh surface produces an immediate blue coloration only in the portion near the skin. Subsequent addition of hydrogen peroxide results in the production of a blue colour of nearly uniform intensity over the whole surface. It is frequently found that if a section of tuber, cut through the centre as in the last instance, be allowed to remain exposed to air for some time before treatment with guaiacum, a more or less uniform coloration over the whole surface is obtained. It is not, however, always a simple matter to demonstrate peroxide production in a plant tissue by this latter method. Moore and Whitley [1909] suggest that the occasional failure of this test is due to the destructive action of excess of oxygen on the peroxide first formed. Since, however, the test is nearly always positive in winter and frequently negative when tissues are examined in the summer, a more likely explanation would seem to be that at a time of relatively great vegetative activity the peroxide is used up as quickly as it is formed.

Various authors have suggested that the difference between the direct oxydase reaction and the indirect, or peroxydase, reaction is one of degree only, depending on the relative concentrations of a single oxidising enzyme. The fact that peroxydase enzyme preparations of very different concentrations are not known to show any difference in their manner of reacting with guaiacum would appear to be strong evidence against this view. When a vegetable extract has been made capable, by exposure to air, of directly causing the oxidation of guaiacum (without special addition of peroxide) the change accomplished is found to have taken place in a constituent other than the peroxydase. The following observations make this point clear.

Some freshly-peeled potatoes or mangold roots are finely minced and the pressed-out juice is treated with not less than five times its volume of rectified spirit. This ensures fairly complete precipitation of the peroxydase. After standing for a day the precipitate is filtered off. The peroxydase contained therein is purified somewhat by dissolving in water and reprecipitating. A solution of peroxydase so obtained gives no coloration when added to fresh guaiacum tincture. If a few cc. of the fresh alcoholic filtrate be diluted with an equal volume of distilled water and then added to the mixture no oxidation of the guaiacum will be found to take place. It will be found, however, on storing the two fractions obtained from the plant extract, in contact with air, that the alcohol-insoluble fraction shows no alteration in the manner of its reacting with guaiacum, while the alcohol-soluble fraction gradually acquires the power of reacting with guaiacum in presence of peroxydase. This alteration which takes place in alcoholic extracts of plants on storing in contact with air would seem to be the most reliable method of demonstrating the formation of peroxide from plant material. The percentage of alcohol (nearly 80 %) in the extracts is such as to eliminate bacterial action.

These results lend very considerable support to the Bach-Chodat view as to the dual nature of the oxidative system of the plants examined. The question arises as to what extent the classification by these authors of the production of peroxide as an enzyme reaction, as ordinarily understood, is justified. An investigation as to the nature of the substance concerned was therefore undertaken.

Nature of the Peroxide-forming Constituent of the Plant-cell.

Moore and Whitley [1909] in a study of oxidising enzymes demonstrated the presence of peroxides in plant juices and their formation by action of atmospheric oxygen. They disputed, however, the intervention of a special enzyme in the production of these peroxides, as had been suggested by Bach and Chodat. Onslow, M. W. [1919, 1920] asserted that the peroxide was produced by a derivative of catechol present in the plant, and that the fixation of oxygen to this body was catalysed by an "oxygenase" also said to be present. Prior to the publication of the Bach and Chodat theory Kastle and Loevenhart [1901] stated that oxydase is not a true ferment, but an organic peroxide. They explained its activity on Baeyer and Villiger's theory in that it acts as a carrier of oxygen in the same way as does benzaldehyde, but is not a true catalytic agent. The Baeyer and Villiger theory referred to is in reality that of Bach [1897]. In dealing with the well-known phenomenon of autoxidation Bach suggested that the substance undergoing autoxidation united with whole molecules of oxygen to form a peroxide. The peroxide thus formed, or the hydrogen peroxide in the so-called nascent state resulting from its decomposition, was held to be the active oxidising agent in such a case, for instance, as the oxidation of indigo simultaneously with the autoxidation of benzaldehyde or of oil of turpentine. A peroxide of benzaldehyde of the type suggested

by Bach was subsequently prepared by Baeyer and Villiger [1900]. It will be noted that Kastle and Loevenhart regarded oxydase as a single active substance. Their statement, that organic peroxides give an immediate blue coloration with guaiacum, could not be confirmed in the present work, other than in the case of benzoyl peroxide. Organic peroxides in general, like hydrogen peroxide, only cause a rapid oxidation of guaiacum in presence of a catalyst, such as various salts of manganese or iron, or in presence of natural peroxydase. The necessity for some such catalyst is at present fairly generally accepted[1].

In connection with the mechanism of the production of peroxide in the plant, it will be seen that the view of Kastle and Loevenhart, in which it is suggested that one of the plant constituents is of an autoxidisable nature is relatively more simple and in keeping with the facts than the assumption of a special ferment. The known autoxidisable organic compounds are invariably substances of an unsaturated nature. The production of a peroxide as an essential stage in autoxidation phenomena, though difficult to demonstrate in some cases, has, none the less, been confirmed in many instances. Thus Engler and Weissberg [1904] have succeeded in preparing, in a pure condition, peroxides of fulvene formed by the action of molecular oxygen. Harries and Muller [1902] describe similar preparations. In many other cases, though the actual peroxide has not been isolated, its presence can be shown by means of suitable reagents. Unsaturated substances present in oil of turpentine give rise to peroxide derivatives in presence of air. As is well known, aerated turpentine may be substituted for hydrogen peroxide in the ordinary guaiacum test for peroxydases. The same is true of aerated benzaldehyde or linseed oil, though in these cases but slight amounts of peroxide seem to be present.

M. W. Onslow [1919, 1920] claims to have shown that the blackening of plant tissues on injury is due to the oxidation of a substance which contains a catechol group, and it is stated that this same phenolic substance functions as the medium of peroxide production in plant tissues. The *ortho*-diphenol concerned has not been characterised further than by the green coloration given by certain plant extracts with ferric chloride. For the purpose of investigating the generalisations mentioned an examination was made in the present work of the root of the common mangold, *Beta vulgaris*. The mangold root, on mincing, first becomes brown and then intensely black on exposure to air. An extract of the root, prepared as described further on (Experimental Part, Section 3) was found to give a blue-black precipitate with a trace of ferric chloride. On adding excess of the salt a green coloration was obtained. On further examination, the extract was found to give many reactions charac-

[1] The guaiacum solution used in the present work was made by dissolving 0·5 g. of translucent resin in 5 cc. of boiling alcohol. An equal volume of water was then added. No coloration should be obtained on addition of a pure peroxydase; nor should any oxidation take place on addition of peroxide in absence of peroxydase. It will usually be found that only fresh solutions of the reagent fulfil both of these conditions.

teristic of the tannins. It is thus evident that the coloration obtained with ferric chloride is due to a substance of this nature. The substance present in the mangold appears to be a gallo-tannin, and the question arises as to whether the catechol derivative referred to above be not, in fact, a catechol tannin.

Peroxide-forming property of Tannin-fraction from the Mangold.

After allowing the tannin-containing extract to remain in the bottom of a stoppered Erlenmeyer flask for a couple of weeks it was found that on adding a quantity to a mixture of the peroxydase enzyme and fresh guaiacum tincture, a slight oxidation of the guaiacum took place. The solution was subsequently stored in a dark place for some months. A black amorphous substance was deposited. After two months the test with a mixture of peroxydase and guaiacum was found to give quite an intense blue coloration. At this stage, however, it was noted that the solution no longer gave any coloration whatsoever with ferric chloride. Quantitative study here will be necessary to determine definitely whether production of peroxide continues after the disappearance of the tannin reactions. It would appear from the qualitative study made that the production of peroxide tends noticeably to increase as the phenolic reaction disappears.

"Anti-oxygen" substances.

It will be seen from the foregoing that the view that catechol derivatives are the peroxide-forming substances of the plant is based on insufficient evidence. The inhibitory influence of phenols on the fixation of oxygen to autoxidisable substances, as recently studied by Moureu and Dufraisse [1922] renders this view of the function of phenols in the plant rather untenable. The results of these authors show that the addition of phenols or tannins to autoxidisable compounds prevents the formation of peroxide, and they ascribe to such substances the rôle of anti-oxydases or oxydation buffers (anti-oxygènes).

The Blackening of Vegetable Juices on Exposure to Air.

When the fresh tannin-containing fraction from the mangold root is mixed with a solution of the peroxydase fraction from the same root the blackening characteristic of the fresh juice is obtained on exposure to air. It was found, however, that the aqueous residue which remained after extraction of the tannin portion with ether-alcohol mixture likewise gave this blackening even to a much stronger degree. It was found on exhaustively extracting this aqueous portion with ether that the substrate of the blackening process still remained in the aqueous layer after the substance which gave the ferric chloride coloration was completely removed. This clearly indicates that the compound which gives rise to the black oxidation products must be other than that which gives the ferric chloride coloration. Closer examination of the blackening process led to the conclusion that the characteristic blackening of the juice of the mangold is, in fact, due to the action of tyrosinase on tyrosine. A study of the darkening of the juice of the potato led to a similar conclusion (see

Experimental Part, Section 2). Bertrand [1896] attributed the blackening of the sap of beetroot, potato and dahlia to the oxidation of tyrosine by tyrosinase.

The Peroxide-forming Constituent of the Potato.

The bulk of the evidence would seem to indicate that the oxygenase of Bach and Chodat is a substance of an autoxidisable nature. Experiments were consequently carried out with a view to the isolation of such substance or substances from the plant. The material chosen for special study was the potato tuber. In this particular isolation there existed the disadvantage that the substance sought for gave no direct characteristic reaction which would enable its location after a series of operations to be rapidly and easily ascertained. It was necessary to adopt the procedure of starting with material which gave no reaction for peroxide when treated with a mixture of fresh guaiacum tincture and peroxydase solution and subsequently carrying out operations as far as possible in absence of oxygen. It was thus possible to ascertain whether any particular fraction obtained during the course of the treatment contained a constituent of peroxide-forming properties by exposing a portion of it in solution to the action of air or oxygen and subsequently testing this with guaiacum-peroxydase mixture.

The experimental details of the method, as finally adopted, are given further on. Briefly, the method consists in finely mincing the tubers and in extracting for 2 hours with alcohol on the water-bath at 50–60°. The product is filtered and evaporated down nearly to dryness under reduced pressure. The aqueous residue in the flask is then extracted with ether. The ether extract is filtered and evaporated down to small bulk. It is then poured into a large excess of acetone. The precipitate having been allowed to settle, the acetone is filtered off. The precipitated material is purified by frequent repetition of the process of precipitation from ethereal solution by means of acetone. The substance thus obtained proved to be of phosphatide nature. The acetone washings from each precipitate were found, after standing some hours in presence of air, to develop the peroxide reaction towards guaiacum-peroxydase mixture. This phenomenon was observed even after repeated precipitation. After removal of all traces of ether and acetone in a vacuum, an alcoholic solution of the substance exposed to air also showed peroxide formation. The final product from the acetone precipitations was precipitated from alcoholic solution as a cadmium chloride double compound. On decomposing this the regenerated lipin continued to give evidence of peroxide formation in presence of air.

Peroxide-forming property of Potato juice associated with the Lipins.

From these results it is evident that the production of peroxide in the potato is intimately associated with the lecithin of the tuber. The substance from which the peroxide is derived may either be the lecithin itself or a compound intimately associated with it. While bearing in mind the latter possi-

bility, it may be pointed out that, generally speaking, lecithins are unsaturated substances owing to their containing unsaturated fatty acids in the molecule, and autoxidation is very generally associated with unsaturation. Erlandsen [1907] gives some extremely interesting details as regards the autoxidisable nature of a lecithin which he examined. The freshly prepared substance proved to have the formula $C_{71}H_{125}NP_2O_{21}$. After storage for some time in dry air it was found to have altered to $C_{71}H_{125}NP_2O_{30}$. Storage of the material for one month in an unevacuated desiccator resulted in an increase in weight of nearly 9 %. The iodine value of the fresh material he found to be 101; after combination with oxygen it was found to have fallen to 22. This change of iodine value appears to indicate alteration of the fatty acid radicles under the influence of atmospheric oxygen. Thunberg [1911] makes the observation that lecithin is oxidised in air by ferrous ammonium sulphate. It would thus appear to form a peroxide after the manner of benzaldehyde (Bayliss).

Function of Lecithins in Respiration.

Palladin and Stanewitsch [1910] pointed out an apparent relationship between the lecithins and plant respiration. They treated wheat embryos with organic solvents and then soaked them for a short time in water, estimating the amount of carbon dioxide evolved during definite intervals. They found that the respiration energy was lowest after treatment with those solvents which extracted most lipin. Vernon [1912, 1914] claims to have demonstrated the dependence of the action of oxydases in the animal organism on the cell lipins. He concluded that the effect of the oxydase is dependent on the lipin membrane which, he suggested, holds together the tissue oxygenase and peroxydase and makes possible their mutual enzymic activity. If, however, as the present research on the potato indicates, the lipin be itself an oxygenase the results of Vernon, as also those of Palladin and Stanewitsch, are readily explicable.

Lecithins are known to be of very general occurrence in animal and vegetable cells, and their function, or functions, has so far remained rather obscure. The property of lecithins of being able to combine readily with molecular oxygen to produce a peroxide would therefore seem to be of rather general importance from the point of view of biological oxidation. Dakin [1922] has pointed out the marked resemblance between oxidation within the animal body and oxidations effected in the laboratory by means of hydrogen peroxide. Kostytschew [1910] claims to have succeeded in oxidising certain degradation products of glucose by the simultaneous action on these substances of a vegetable peroxydase and hydrogen peroxide. Free hydrogen peroxide appears to be practically absent from both vegetable and animal tissues. It is known to be relatively toxic to both, and the ferment catalase, of very general occurrence, prevents its accumulation. According to Bach and Chodat [1903, 2] catalase is entirely without action on "oxygenase" or on ethyl hydroperoxide. It is thus evident that a substituted peroxide of the type of ethyl hydroperoxide

must be the active oxidising agent in the cell rather than free hydrogen peroxide. Such peroxides, as a rule, are found to be very weak oxidising agents and require the addition of a catalyst before oxidations such as that of guaiacum, benzidine, etc., can take place at all readily. In the plant the catalyst is usually a peroxydase. It yet remains to be definitely determined whether the lecithin of animal tissues is capable, by the production of peroxide, of causing the oxidation of other substances present[1]. The well-known peroxydase reaction of haemoglobin with respect to guaiacum, benzidine, etc., is of interest in this respect, and suggests that haemoglobin or some closely related substance in animal tissues may be capable of fulfilling a catalytic rôle comparable to that of peroxydase in the plant.

Plant Constituents other than the Lipins which may function as "Oxygenases."

Unsaturated substances, as has already been pointed out, are very frequently found to be autoxidisable. It is thus reasonable to expect that unsaturated compounds, other than the lipins, present in a plant may, according to circumstances, function as "oxygenases." The formation of peroxide by oil of turpentine, itself a plant product, is well known. It appeared interesting, therefore, to submit to examination a number of other terpenes with a view to ascertaining whether they behave in a similar manner. Specimens supplied by British Drug Houses, Ltd., were examined as delivered. The following gave a strong blue coloration with guaiacum-peroxydase mixture: limonene, cumene, cedrene, phellandrene, terpineol, terebene, terpinol, linalol and carvone. Pinene and carvene gave no coloration on preliminary examination. On placing a small quantity of each in test-tubes and passing a current of oxygen through for about four hours they were found, however, to have acquired the property of causing the oxidation of guaiacum in presence of peroxydase. On the other hand, samples of caryophyllene, citral, citronellal, camphene and terpene hydrate, even after oxygen had been passed through them for eight to nine hours, gave scarcely any coloration with guaiacum-peroxydase mixture. Like pinene and carvene, a specimen of oleic acid gave no reaction for peroxide until oxygen had been bubbled through it for three to four hours. The production of peroxide by oleic acid is of especial interest in view of its frequent occurrence in the molecule of lecithin-like substances.

These observations on the occurrence of peroxide among the terpenes examined are provisional. It is possible that in individual cases where a positive reaction for peroxide was obtained, the reaction may be due to an autoxidisable impurity present; or on the other hand, in those instances where no peroxide appears to be formed, the negative result may be due to an inhibiting substance. The results as given are none the less interesting as indicating the ease with which the terpenes, as a class, give rise to peroxides. It appears quite likely, in consequence, that these substances are thus capable of taking part in the respiration of the plants in which they are found.

[1] A specimen of egg-lecithin in alcoholic solution was found, after several days' exposure to air, to acquire the power of oxidising guaiacum in presence of peroxydase.

Experimental Part.

1. *Preparation of Solutions of Peroxydase from the Mangold root and the Potato.*

The solutions of peroxydase employed in the course of the present work were invariably prepared from the root of the mangold. The most suitable variety of root for the preparation is that known as "yellow globe." The product obtained from the mangold is not a pure peroxydase, but contains a considerable proportion of tyrosinase. The same is true of that from the potato tuber. It would appear that most tyrosinase preparations so far studied give the peroxydase reaction with guaiacum, but whether this is due to admixture with ordinary peroxydase or a property of tyrosinase itself it is impossible to say.

For the purpose of preparing the enzyme solution the plant material is first finely minced. The juice is then pressed out from the minced material through a double layer of fine muslin, and is treated with about five times its volume of rectified spirit. The mixture is allowed to stand overnight. The precipitate which collects is then filtered off. It is purified somewhat by redissolving in water and reprecipitating the enzyme by the addition of more alcohol in approximately the same proportion as above. The precipitate obtained in this case is likewise allowed to settle. After again filtering off, it is dissolved in a quantity of distilled water approximately equivalent to one-fifth the volume of the original juice.

The solution of peroxydase thus obtained from the mangold root, or from the potato, is a clear liquid of slight brown colour. In presence of hydrogen peroxide it oxidises guaiacum, guaiacol, benzidine, *p*-phenylenediamine, α-naphthol, etc. In the absence of hydrogen peroxide it is without action on these substances. A mixture of peroxydase and one of these substances is consequently an excellent reagent for detecting hydrogen peroxide, either in the free condition, or combined as a peroxide derivative. In the course of the present work use has been made of a mixture of peroxydase solution and fresh guaiacum tincture for this purpose. For this test to be reliable it is essential that care be taken to use a guaiacum solution free from peroxide, otherwise the mixture of guaiacum solution and peroxydase alone will yield the characteristic blue colour. It is likewise essential to note that the substance under examination be incapable of oxidising guaiacum in the absence of peroxydase[1].

[1] Guaiacum is readily oxidised by ozone, nitrogen peroxide, free nitrous and nitric acids, permanganates and chromates. In each of these cases, however, the action takes place in the absence of peroxydase. In the case of solutions, therefore, where guaiacum is oxidised only when peroxydase is also added there is ample evidence for believing that the solution contains either hydrogen peroxide or other peroxides of similar constitution. The guaiacum reaction, as described, would thus appear to be a specific test for peroxides. This cannot be said of the potassium-iodide-starch test which has been commonly employed in dealing with these substances. Guaiacum-peroxydase mixture appears to be considerably more sensitive to organic peroxides than mixtures of peroxydase and other common oxydase reagents (such as benzidine etc.) which are sometimes employed.

2. *Blackening of the Juice of the Mangold and of the Potato due to the action of Tyrosinase on Tyrosine.*

The influence of tyrosinase on the blackening of the juice of the potato may be demonstrated as follows.

A quantity of potato tubers is finely minced and the pressed-out juice is quickly filtered or centrifuged. About 10 cc. of the clear juice is then transferred to a boiling-tube by means of a pipette, care being taken that the juice falls directly to the bottom and does not spill along the sides of the tube. The boiling-tube is then immersed in a water-bath at 75° and the juice is stirred with a thermometer. When the juice has attained the temperature of the water-bath the time is noted and heating is continued for 10 minutes at this temperature. The contents of the tube are then cooled.

It will now be found that although the contents of the tube still give a strong peroxydase reaction with guaiacum, no darkening in colour takes place on exposure to air. If a quantity of tyrosine or of *p*-cresol be added to a portion of the juice so heated, the characteristic colorations given by these substances in presence of tyrosinase are no longer obtained, although fresh potato juice gives this reaction. It is thus evident that the tyrosinase has been destroyed on heating. If now a solution of tyrosinase, prepared in the ordinary manner from any plant containing it, be added to another portion of potato juice which has been heated as described, the darkening in colour characteristic of fresh potato juice is again obtained. Addition of a pure peroxydase preparation, such as that from the horse-radish, produces no change. That the actual substrate in this blackening process in the case of potato juice is free tyrosine is rendered very probable by the isolation from the potato of considerable quantities of this amino acid in the free condition. The substance was isolated incidentally in another portion of this work (see Section 6, Experimental Part) and considering that it was obtained in an alcohol-ether extract of a concentrate of the juice, the yield of 0·3 g. from $2\frac{1}{2}$ kilos. of fresh tuber is very probably far from quantitative.

A similar destruction of the tyrosinase in the mangold root results in the prevention of the blackening of the juice. The tyrosinase of the mangold is usually found to be somewhat more resistant to heat than that of the potato, and a rather longer period of heating than 10 minutes at 75° is found necessary to complete its destruction in some samples. The peroxydase is also relatively more resistant to heat in this case than in that of the potato.

3. *Study of the Tannin of the Mangold root.*

The alcoholic filtrates obtained in the preparation of the peroxydase of the mangold were now studied in connection with the suggestion made by Onslow, M. W. [1919, 1920] that the peroxide-forming constituent in the plant as well as the substrate in the blackening process of the juice was a derivative of catechol. The alcoholic solution (six litres) obtained after removing

the peroxydase-tyrosinase fraction of the juice was concentrated *in vacuo* at 40–50°. When all of the alcohol had been removed and the aqueous residue suitably reduced in bulk the distillation was stopped. The residue was then extracted with ether containing about 20 % of alcohol. The ether was distilled off from the extract. A residue consisting of a solution in a mixture of water and alcohol was thus obtained. With ferric chloride it gave a blue-black coloration or precipitate, which disappeared on adding excess of the salt, yielding a green solution. On submitting the extract to further examination the following reactions were observed:

(i) Addition of alkali causes the solution to darken in colour.

(ii) With a solution of ammonium picrate it gives a red colour, which changes to green.

(iii) On adding a little sodium sulphate, and then treating with a dilute solution of iodine a purple-red colour results.

(iv) With a solution of potassium cyanide scarcely any change takes place.

(v) A purple colour is obtained on adding, first, ammonia, and then nitric acid, to the solution.

(vi) On treating 2 cc. of the solution with three drops of 20 % thymol solution and then adding 3 cc. of strong sulphuric acid a deep red coloration is obtained.

(vii) Lime-water gives a greyish precipitate which rapidly turns blue.

These reactions are characteristic of the tannins, and it is thus evident that the coloration given by ferric chloride is due to a substance or substances of this type. It is interesting to note, moreover, that tannins, as a rule, are said to have an inhibiting influence on oxydase action.

4. *Peroxide-forming character of Alcoholic Extract of Plants, and Action of Phenols thereon.*

The production of peroxide by a substance in plant tissue may best be demonstrated by exposing an alcoholic extract of the tissues to air. An alcoholic extract for the purpose may conveniently be prepared as follows.

Fresh mangold roots, or a quantity of potato tubers, are peeled and then finely minced. The minced material is quickly strained through several layers of muslin, 500 cc. of the juice thus obtained are then added to 2 litres of rectified spirit. The mixture is well shaken and after being allowed to stand overnight, is filtered. The alcoholic extract thus obtained produces no change when added to a mixture of peroxydase and fresh guaiacum tincture. In making the test 5 cc. of the alcoholic plant extract is diluted with an equal volume of distilled water before adding it to a mixture of peroxydase, prepared as in Section 1, and a few drops of a 5 % solution of guaiacum in 50 % alcohol. The dilution of the extract with water is necessary to prevent precipitation of peroxydase. On storing the alcoholic extract in presence of air, no change in its behaviour relative to a guaiacum-peroxydase mixture is observed till after a certain length of time. In the case of an extract of potato, 50 cc. of solution in an uncorked

250 cc. flask, placed so as to be exposed to daylight, only began to oxidise guaiacum-peroxydase mixture after two days' exposure. An extract of the mangold exposed in a similar manner required over a week's exposure before the presence of peroxide could be detected. There is thus evidence of a certain latent period in the production of peroxide, and this is probably due, in part at any rate, to the presence of phenolic substances such as tannins. The inhibiting influence of such substances on the course of autoxidation has been demonstrated by Moureu and Dufraisse [1922]. In the course of normal plant metabolism the proportion of these inhibiting substances present is probably controlled by the peroxydase, the known oxidising power of the latter enzyme relative to phenolic substances being responsible for its being commonly classed as phenolase.

The rate at which peroxide is produced in the plant alcoholic extracts appears to be very much accelerated by the action of light. Light, however, is not essential to the production of peroxide, since extracts stored in the dark were also found to have acquired it. As might be expected, the free admission of oxygen has also a marked accelerating influence.

5. *Action of the "Anti-oxygènes" of Moureu and Dufraisse on the production of Peroxide in Plant extracts.*

The following table shows the influence of traces of some common phenols on the rate of peroxide formation in alcoholic extracts of the mangold, prepared as described in Section 4.

100 cc. alcoholic extract treated with phenol in proportion of 1 : 100,000	Test with guaiacum-peroxydase mixture after one week's exposure to air	Test with guaiacum-peroxydase mixture after two weeks' exposure to air
1. Quinol	No coloration	Faint blue coloration
2. Gallotannic acid	,,	,,
3. Pyrocatechol	Blue coloration	Very strong blue coloration which quickly faded
4. Control	,,	Strong blue coloration

The inhibiting influence of traces of quinol and of gallotannic acid on peroxide formation is seen to be distinctive. That the action of these substances is to prevent the production of peroxide is shown by the fact that subsequent addition of peroxide to the solutions containing them, after treatment with guaiacum and peroxydase, gives rise to the usual intense blue colour. Pyrocatechol appears to be without inhibiting action in the dilution employed. In the case of potato extract, *p*-cresol was likewise found to be without inhibiting action when added in traces similar to the above. On the other hand, a trace of pyrogallol was found to be quite effective in preventing peroxide formation.

6. *Preparation of the Peroxide-forming Constituent of the Potato.*

The method by which this substance was first prepared consisted in finely mincing $2\frac{1}{2}$ kilos. of freshly-peeled potato tubers. The minced material was then added to $7\frac{1}{2}$ litres of rectified spirit and the mixture was allowed to digest in the cold for 24 hours. The solid matter was then filtered off on the Buchner

funnel. The filtrate was evaporated down *in vacuo* at 40–50° until 400–500 cc. of aqueous residue remained. A quantity of waxy material separated. This was removed by thorough extraction with ether. This ether extract was subsequently found to contain the bulk of the peroxide-forming constituent of the tuber. The aqueous portion remaining after the ether extraction was then shaken with a mixture of equal parts of alcohol and ether. On concentrating this extract somewhat, a yield of 0·3 g. of free tyrosine was found to separate. That this amount of tyrosine is by no means indicative of the total amount of this amino acid present in the original tuber is evident from a consideration of the solubility of tyrosine in the solvents employed, as also from the fact that tyrosine continued to separate slowly, in a rather impure condition, from the aqueous solution remaining after the extraction. The ether extract obtained above from the original concentrate was then worked up. On distilling off the ether a wax-like material was obtained. This was redissolved in 15–20 cc. of ether. On addition of about 250 cc. of alcohol a light yellow solid was precipitated. The precipitate was redissolved in a small quantity of ether and then poured into about a litre of acetone, with stirring. A precipitate, more nearly colourless than that given by alcohol was thus obtained. On allowing the acetone washings from this material to remain exposed to air for some hours and then testing them with peroxydase-guaiacum mixture a blue colour resulted, indicative of the formation of peroxide in the liquid. The precipitate obtained with acetone was again redissolved in a small quantity of ether and again poured into a large excess of acetone (500–600 cc.). This method of purification was repeated six times. In each case the acetone washings continued to give a peroxide reaction after exposure to air for some time. The final product, on drying off the acetone, became quite brown in colour, and contracted to a wax-like mass. On drying it in a vacuum desiccator it became quite brittle and could be easily reduced to powder. It proved to be relatively slightly soluble in alcohol. In alcoholic solution production of peroxide was also noted. About 0·2 g. of the substance was dissolved in alcohol by warming to 50–60°. 5 cc. of a saturated alcoholic solution of cadmium chloride were added. After standing for some time the precipitate which separated was filtered off. It was placed in a beaker and about 20 cc. of ether added. After acidifying with a drop of 10 % hydrochloric acid, the cadmium was precipitated by hydrogen sulphide and the suphide was filtered off. Excess of hydrogen sulphide was then removed with a current of air and the ethereal solution remaining was washed by shaking in a separating funnel, first with a saturated aqueous solution of sodium bicarbonate and finally with distilled water. The remaining ethereal solution was then poured into excess of acetone. The product thus obtained continued to show peroxide-forming properties. After prolonged action of air it was found capable of liberating iodine from potassium iodide, even in the absence of acid.

It is evident from these results that the formation of peroxide is due to the autoxidation of a lecithin-like substance. It has been pointed out by

various workers on plant lecithins that these substances are not very readily extracted from the tissues by cold solvents. Schulze and his co-workers, who investigated many lecithins of this type, carried out their extractions with alcohol at 50–60°. The extraction of a further quantity of lecithin from the potato tuber was accordingly carried out as follows.

Three kilos. of fresh, finely minced tubers were treated with five litres of 90% alcohol and the mixture was digested on the water-bath for two hours. To prevent autoxidation at this temperature a current of nitrogen was passed through the liquid during the digestion. The extract was then pressed out from the solid material through a couple of layers of fine muslin. The liquid thus obtained was cloudy, owing to solid matter in suspension. On allowing it to stand for about a quarter of an hour the solid material readily settles down. The liquid may then be decanted and the remainder filtered on a Buchner funnel. The liquid was evaporated down as in the previous preparations and the residue was then thoroughly extracted with ether. After dehydrating the extract with anhydrous sodium sulphate the liquid was filtered and the greater part of the ether was distilled off. The ethereal solution remaining (about 50 cc.) was then added to a litre of acetone. The liquid was allowed to stand in the dark for about an hour and the lecithin which separated out was then filtered off and dried in a vacuum desiccator. The yield of lecithin thus obtained from three kilos. of fresh tuber was 1·6 g. The substance thus obtained may be further purified as in the previous preparation.

A specimen of the substance obtained in the present work was submitted to hydrolysis for the purpose of characterisation. 0·5 g. of the substance was boiled for five minutes with 12·5 cc. of 6% sodium hydroxide solution. 2 cc. of glacial acetic acid were then added, and boiling was continued for a further minute. The acid liquid was allowed to cool. It was filtered through a moistened filter paper. The presence of phosphate in the filtrate was proved by precipitates given by ammonium molybdate and magnesium sulphate. By treatment with a solution of iodine in potassium iodide, no evidence of the production of "Florence crystals" said to be characteristic of choline [Struve, 1900] could be obtained, but microscopic crystals unlike those obtained from choline were precipitated. These may possibly be due to betaine, which substance is said to replace choline in some plant phosphatides. It is hoped in further work to continue the study of this phosphatide.

Summary.

The present work deals with the nature of the "oxygenase" in the Bach and Chodat theory.

It is shown that plant juices, on exposure to air, form peroxides. Alcoholic extracts of plant tissues, containing a sufficiently high content of alcohol to prevent bacterial action, are found to serve best for demonstrating this production of peroxide.

The formation of peroxide by the action of atmospheric oxygen on plant

extracts appears to be markedly influenced by the addition of certain phenolic substances such as quinol or gallotannic acid. Minute traces of such substances appear usually, though not always, to prevent the fixation of oxygen.

The suggested relation of catechol to the plant oxydase system is discussed. It is shown that the blackening of aqueous extracts of the potato and of the mangold is due, not to the oxidation of a catechol derivative under the influence of peroxydase, but to the action of tyrosinase on tyrosine. A detailed examination of the extract of the mangold root which gave a ferric chloride coloration revealed the fact that the reactive substance present was a tannin. The tannins, in general, are said to have an inhibiting influence on oxydase action.

There appears to be no definite evidence that the production of peroxide in the plant is due to enzyme action; it seems more likely that the formation of peroxide is due to the presence of an autoxidisable substance in the tissues. A substance of this nature isolated from fresh potato tubers was found to bear a close relation to the lipins. In contact with air or oxygen a solution of this substance acquires the property of causing the immediate oxidation of guaiacum in presence of peroxydase. It would thus appear that the so-called oxygenase of the potato is in reality an autoxidisable lecithin-like substance.

A study of the terpenes as possible "oxygenases" in the plants in which they occur would be of interest. It is shown that these compounds as a class are capable of combining with oxygen in such a manner as to cause the oxidation of guaiacum in presence of peroxydase.

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LXIV. THE PENETRATION OF ELECTROLYTES INTO GELS.

V. THE DIFFUSION OF MIXTURES OF CHLORIDES IN GELS.

By WALTER STILES.

From the Botanical Laboratory, University College, Reading.

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Introduction.

Considerable emphasis has been laid during recent years on the analogies between colloidal systems and living cells in regard to their behaviour towards water and dissolved substances and it has become evident that the study of colloidal systems must throw light on the constitution and behaviour of living tissues. It is therefore of interest to enquire whether anything analogous to the phenomenon of antagonism between ions in their entrance into living tissues is exhibited in the penetration of electrolytes into gels.

Among the many antagonistic actions recorded for various plant tissues, those between different chlorides were among the earliest, and are perhaps now the best, known. Thus a very definite antagonism between sodium and calcium chlorides, between potassium and calcium chlorides, and between potassium and magnesium chlorides, and even between chlorides of the univalent metals, has been recorded by different writers [see Stiles, 1923, 1]. Experiments were therefore made on the diffusion of mixtures of chlorides through gels of agar-agar and gelatin with a view to determining whether any mutual hindrance to the penetration of salts through the gel resulted from the presence of one salt along with the other, such as might be compared with the antagonistic effects observed in living systems.

Method.

The measurements were made by means of the indicator method already described [Stiles, 1920; Adair, 1920]. In the earlier experiments, carried out four years ago, the original crude "half test-tube" method was employed. Subsequently, the refined technique recently described in detail [Stiles, 1923, 2] was employed, and coefficients of diffusion calculated from the measurements made.

Two gels were used: a 1 % agar-agar gel made from shred agar-agar, and a 10 % gelatin gel. In both cases the material was washed with distilled water before use until the washing water was free from chloride. The gels contained silver nitrate as indicator, which was present in a concentration of 0·005*N*.

The earlier experiments were conducted in a dark cupboard in the laboratory without temperature control, but the results obtained in any one series are strictly comparable as all the experiments in a series were carried out together. The latter was also the case with the later more exact experiments, but these were carried out in water-baths maintained at constant temperature by means of gas-mercury-toluene thermo-regulators.

Experiments were conducted with a number of pairs of chlorides, the two chlorides of the pair being present in a variety of proportions, but in all cases the solutions were normal as regards chloride. In the calculation of the coefficient of diffusion the solutions are assumed to be normal solutions, as they are such in regard to chloride, and Adair's second and more exact formula is used. For the purposes of the calculation the coefficient of diffusion of silver nitrate in the gel is taken as 10^{-5} sq. cm. per second [cf. Adair, 1920]. An error of 50 % in this does not alter appreciably the final values obtained for the coefficients of diffusion of the chlorides.

Experimental Results.

1. *Agar-agar.*

The diffusion of the following mixtures was examined by the half test-tube method: sodium chloride and calcium chloride, potassium chloride and calcium chloride, potassium chloride and magnesium chloride, and potassium chloride and sodium chloride. The experiments were carried out in duplicate. Each tube contained a length of 7 cm. of gel surmounted by the same length of solution of diffusing salt. Solutions of the pure salts and solutions containing varying relative proportions of the two chlorides were used, but in no case could any indication of a hindrance to diffusion of the salt in a mixed solution be discovered. The penetration was measured after a number of different times; the results obtained for measurements made after diffusion had proceeded for about 27 hours are recorded in Table I. Similar results were obtained after both shorter and longer periods.

Table I. *Penetration of chlorides into gels of* 1 % *agar-agar from pure and mixed solutions.*

Relative proportions of chlorides First named : second named	Penetration in centimetres			
	$NaCl + CaCl_2$ 27·3 hrs.	$KCl + CaCl_2$ 27·8 hrs.	$KCl + MgCl_2$ 27·25 hrs.	$KCl + NaCl$ 27·63 hrs.
10 : 0	4·19	4·60	4·59	4·59
9 : 1	4·15	4·525	4·50	4·56
8 : 2	4·15	4·525	4·49	4·52
7 : 3	4·165	4·505	4·43	4·51
6 : 4	4·09	4·43	4·40	4·49
5 : 5	4·08	4·41	4·32	4·42
4 : 6	3·965	4·32	4·29	4·42
3 : 7	3·95	4·23	4·15	—
2 : 8	3·94	4·13	4·09	4·345
1 : 9	3·81	4·01	3·915	—
0 : 10	3·76	3·79	3·655	4·21

Although the method does not permit of the calculation of the coefficients of diffusion, and although the measurements on which each value for the penetration is based are few in number, it is clear from these results that there is no antagonism between the chlorides employed in regard to their diffusion through agar-agar. Nevertheless one series of experiments, that in which mixtures of calcium and sodium chlorides were employed, was repeated by the more exact method and the coefficients of diffusion calculated in each case, assuming that each solution was a normal chloride solution. The values for the coefficient of diffusion are recorded in Table II. They confirm the conclusion drawn from the experiments with the cruder "half test-tube" method as to the absence of any hindrance offered by the presence of one chloride to the diffusion of another, for as the proportion of calcium chloride in the mixture is increased there is a progressive lessening in the value of the coefficient of diffusion.

In order to indicate more clearly the relation between the composition of the solution and the diffusivity the results obtained are shown graphically in

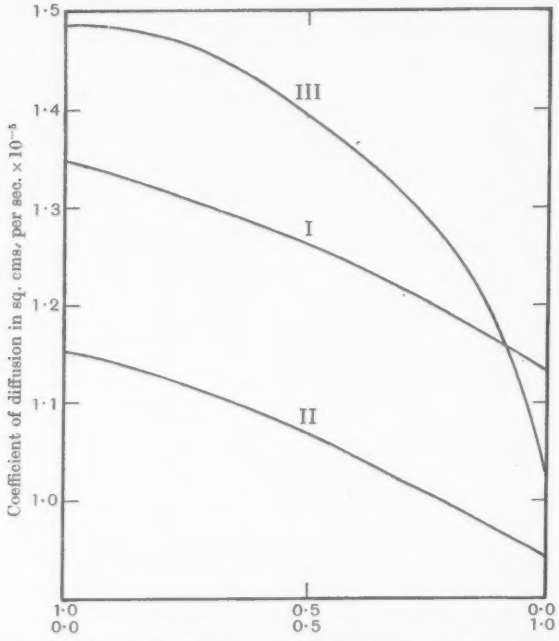

Fig. 1. Curves to illustrate the diffusion through gels of chlorides in pure and mixed solutions
I. Sodium and calcium chlorides in 1 % agar-agar.
II. Sodium and calcium chlorides in 10 % gelatin.
III. Potassium and calcium chlorides in 10 % gelatin.

Fig. 1. These results thus expressed show that so far from there being anything in the nature of a hindrance to diffusion in a mixed solution, the diffusivity in a mixed solution is actually slightly greater than would be expected from the values of the diffusivity of the pure chlorides, as the curve connecting the value of the coefficient of diffusion with the composition of the diffusing solution is concave to the axis of the composition of the diffusing salt and not a straight line.

2. *Gelatin.*

Determinations of the coefficient of diffusion of sodium and calcium chlorides and of mixtures of the two in 10 % gelatin were made by the more exact method, the temperature of experiment being 21·5° ± 0·5°. A similar series of experiments was carried out with potassium chloride and calcium chloride as diffusing salts at a temperature of 21·5°. As before, all the solutions employed were normal in regard to chloride. The results are shown in Table II, and also graphically in Fig. 1. The same phenomenon is obvious here as in the diffusion of mixtures of chlorides through agar-agar, namely, that the rate of diffusion of the mixed chlorides is always a little higher than would be expected from the values of the coefficients of diffusion of the respective salts in pure solutions.

Table II. *Penetration of chlorides into gels of agar-agar and gelatin from pure and mixed solutions.* (*Concentrations of chloride always normal.*)

	Coefficient of diffusion in sq. cm. per sec. $\times 10^{-5}$		
Relative proportions of chlorides First named : second named	$NaCl + CaCl_2$ in agar-agar 20·5°	$NaCl + CaCl_2$ in gelatin 21·5° ± 0·5°	$KCl + CaCl_2$ in gelatin 21·5°
10 : 0	1·347	1·151	1·488
10 : 1	—	—	1·476
9 : 1	1·334	1·141	—
10 : 2	—	—	1·481
8 : 2	1·317	1·131	—
10 : 4	—	—	1·457
7 : 3	1·297	1·106	—
5 : 5	1·269	1·073	1·393
3 : 7	1·210	1·012	—
4 : 10	—	—	1·305
2 : 10	—	—	1·239
1 : 9	1·166	0·969	—
0 : 10	1·130	0·940	1·024

It does not appear easy to find a simple explanation of these results. Although the coefficient of diffusion no doubt increases with increase in the relative number of electrolytically dissociated molecules and hence with dilution, as has been shown to be the case for gels [Stiles, 1923, 2] as well as for free diffusion in water, and although both of the two constituent salts in any one of the mixtures employed are in less than normal concentration, yet the presence of the other salt will depress the degree of dissociation so that an appreciable rise in the value of the coefficient of diffusion is scarcely to be expected on this ground.

A decrease in viscosity of the gel would result in an increase in the rate of diffusion, but the results obtained bv Fenn [1916], if rightly interpreted, suggest that in mixtures of salts such as those employed in the present investigation, the viscosity is greater than in pure salt solutions of the same concentration.

In the present state of our knowledge it would not be profitable to discuss this matter further. It is, however, clear from the investigation here recorded that the antagonistic effects observed with living tissues cannot be attributed to mutual interference between the antagonising salts in their diffusion through any gel layer in the cells of the tissues.

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LXV. BROMOXYLENOL BLUE.
A TRUE-NEUTRALITY POINT INDICATOR.

By ABRAHAM COHEN (*London*).

(*Received June 17th, 1923.*)

In a previous article on xylenol blue, synthesised, and described in this Journal, [1922, 1] by the writer it was shown that the double working ranges of utility coincide with those of thymol blue. The pair of indicators thus share with certain other pairs of homologous indicators the property of anomalous unshifted p_H ranges. Advantage was taken of this property in the case of xylenol blue to obtain a solution of twice the intensity of thymol blue.

When the p_H range of the bromo-derivative of xylenol blue was investigated in the same manner as that of the unbrominated indicator, and compared with bromothymol blue in identical concentrations and strengths of indicator in buffer solutions of p_H 6·0–7·6, the series in each case was similar. The analogy of xylenol blue with thymol blue in respect of range of p_H is thus continued on bromination.

It is easier, however, to brominate xylenol blue than thymol blue, and this was accomplished by adding in the cold, with constant shaking of the reaction flask, 3·1 parts bromine to 1 part of xylenol blue suspended in 10 parts glacial acetic acid. After standing overnight, the pale pink crystals were thoroughly drained by suction and recrystallised from dry boiling toluene, in which the product is much more soluble than the similarly prepared bromocresol purple. It separated white and crystalline on cooling. Submitted to analysis, the product was found to be a *dibromoxylenol sulphonephthalein*.

0·3400 g., fused with caustic potash and sodium peroxide, gave 0·2250 g. silver bromide and 0·1432 g. barium sulphate.

S	5·76 %;	calculated for	$C_{23}H_{20}O_5Br_2S$	5·63 %
Br	28·2 %	,,	,,	28·15 %

Bromoxylenol blue, like bromothymol blue, can have its working range halved by the addition of bromocresol purple, as shown in an earlier paper by the writer [1922, 2]. The resulting mixed indicator can then be used where a blue end-point of Sörensen value about 6·8 is desired in a titration.

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LXVI. BLOOD ENZYMES. III. ON THE GLYCOGENOLYTIC ACTIVITY OF MAMMALIAN SERA—WITH REMARKS ON SERUM TOXICITY.

By ARTHUR COMPTON.

From the "Laboratoire de Chimie Biologique," Institut Pasteur, Paris; the Laboratories of the Imperial Cancer Research Fund, London; and the Surgical Unit, St Mary's Hospital.

(*Received June 20th, 1923.*)

The present communication treats of the enzymes, or enzyme complex, of the blood responsible for the breaking down of glycogen by hydrolysis. The end-products of glycogen hydrolysis being either maltose or glucose, according to the hydrolytic agents employed, it is assumed that at least two distinct enzyme systems are involved in the complete breaking down of glycogen to glucose: one, *glycogenase*, whose function would be to transform the glycogen first into maltose; and the other, *maltase*, concerned with the transformation of the maltose into glucose.

Now, it has been shown by us [Compton, 1921] that mammals differ considerably, from the species point of view, in the content of their blood in the last-mentioned ferment. For instance, when tested *in vitro*, the sera of man, the cat, the rabbit and the guinea-pig are entirely devoid of maltose splitting power; whilst sera from the dog, the horse, the ox, the goat and the pig—particularly the last-mentioned mammal, the blood of which is very rich in the ferment—normally contain *maltase*. Thus it comes about that, in terms of the enzyme content of their blood, there are two groups of mammals: a *positive blood maltase* group, and a *negative blood maltase* group.

On the other hand, the sera of all mammals which we have thus far investigated are capable of breaking down glycogen, with the production of either maltose or glucose. Whether the end-product of the hydrolysis of glycogen by mammalian blood serum is glucose, or maltose, must evidently depend upon the blood maltase group to which the animal belongs.

The results which follow concern the hydrolysis of glycogen by the sera of various mammals in a 16 hours' action at approximately 46–47°, the mammalian species studied comprising representatives of the two groups previously defined by us: (1) the *maltase* group: the dog, the horse, the ox, the pig, and the sheep; and (2) the *non-maltase* group: the cat, the rabbit, the monkey, and man.

It will be seen from the results set forth that here again, as in the case of *maltase*, mammals differ among themselves in the content of their sera in *glycogenolytic power*—as measured by the amount of glycogen hydrolysed in a given time at a given temperature by a given quantity of serum—the activity, moreover, being fairly characteristic of the species.

Experimental.

Preparation of Glycogen. Twenty-two rabbits were fed during a week largely on carrots, and the animals were then killed. The livers were removed immediately, minced, and plunged into an equal weight of 60 % potassium hydroxide, and the mixture heated up to boiling for 5 hours. An equal volume of water was added, and the entire mixture allowed to cool. Contrary to the methods usually described, it was now precipitated directly without previous filtration, by the addition of one and a half volumes of alcohol. After standing overnight, the precipitate was collected by centrifugation, and washed well with 70 % alcohol. The resulting, fairly white, precipitate was now heated on the water-bath for 1½ hours with about six times its own weight (moist) of water to dissolve it, and was then filtered by suction while still hot through two layers of starch-free filter paper on a Buchner funnel. The solution thus obtained was rapidly cooled under the water-tap, and neutralised with concentrated hydrochloric acid, and about 3–5 cc. excess of acid added to precipitate any extraneous protein matter. The solution was next filtered anew through a Buchner funnel, an olive-green residue and a whitish opalescent solution resulting. The filtrate, and washings, some 2¼ litres in all, were poured into 4 litres of 95 % alcohol and the mixture allowed to stand overnight, the resulting white flocculent precipitate being collected and washed with 60 % alcohol. The precipitate (moist) was redissolved in ten times its weight of distilled water, and precipitated a third time with 1½ volumes of alcohol, the opalescent solution being poured into the alcohol. Next morning, the precipitate was collected by centrifugation, washed twice with 70 % alcohol, and then with absolute. It was now dried in a vacuum over sulphuric acid. 120 g. of the dried pure product were thus obtained.

Complete Hydrolysis of Glycogen—its Glucose Content. According to Pflüger [1904], hydrolysis by 2·2 % hydrochloric acid on the water-bath for 3 hours completely transforms glycogen into glucose, and he utilised this as a method for estimating glycogen in tissues. In Pflüger's method the weight of glucose found after hydrolysis is multiplied by 0·927 to find the corresponding quantity of glycogen.

30 mg. of the foregoing specimen, when completely hydrolysed by prolonged heating with 2–3 % hydrochloric acid on the water-bath gave as the result of four experiments the following glucose numbers in mg., the latter being estimated by Bertrand's method: 29·8, 28·6, 29·2, 28·4—mean 29·0. This signifies that 100 mg. of the specimen when completely hydrolysed would yield 96·7 mg. approx. of glucose. From this it follows that 96·7 mg. of glucose found, as the result of the enzymic hydrolysis of 100 mg. of the specimen, corresponds to 100 % hydrolysis when the end-product is glucose. In other words, under such conditions 1 mg. of glucose found represents $\frac{100}{96{\cdot}7} = 1{\cdot}034$ % hydrolysis.

Again, since 1 mg. of glucose is the equivalent of $\frac{342}{360} = 0{\cdot}95$ mg. of maltose, it will be evident, when the end-product is maltose that 1 mg. of maltose found as the result of the enzymic hydrolysis of 100 mg. of the specimen will represent a percentage hydrolysis of $\frac{100}{97{\cdot}6 \times 0{\cdot}95} = 1{\cdot}09$ approx.

Method of Procedure. The method of determining the glycogenolytic activity of the different sera investigated was as follows: 100 mg. of glycogen were carefully weighed out into each of four, or more, clean dry test-tubes, and after adding 2 cc. of redistilled water to each tube, the latter were heated for about 2 minutes in boiling water to dissolve the glycogen. The tubes were rapidly cooled and the contents of each completed with water and varying doses of serum to 5 cc. Three drops of toluene were added, the tubes plunged into a water-thermostat regulated about 46–47°, and the tubes having acquired the temperature of the thermostat were closed with clean sterile corks. After incubation for 16 hours, the corks were removed and the enzyme action stopped by heating for 7 minutes in boiling water. When cold, the contents of each tube were diluted to 50 cc., and 20 cc. were withdrawn for a sugar determination by Bertrand's method. The percentage of glycogen hydrolysed was then calculated in terms of glucose, or of maltose, by the use of one or other of the preceding factors, according as the serum under investigation appertained to a positive, or a negative blood maltase group of mammals.

Blood Maltase Positive Group.

Under this category, sera from the dog, the horse, the pig and the sheep were investigated.

Dog. Three dogs were investigated (2–*P*, 3–*P* and 4–*P*). The animals were, as a rule, in the fasting condition for 24 hours previous to withdrawal of the blood for testing. The animal being immobilised, the blood was collected from the jugular vein into a sterile syringe, and centrifuged immediately to obtain serum for the tests.

The animal 2–*P* was examined, as to the richness of its blood in glycogenolytic activity, on three separate occasions; 3–*P* twice; and 4–*P* twice. The results obtained are set forth in Table I:

Table I.

	Glycogen hydrolysed %, by the blood serum of the following dogs:						
	2–*P*			3–*P*		4–*P*	
Dose of serum employed	1	2	3	1	2	1	2
0·1	29·2	23·7	—	23·7	—	33·0	—
0·15	—	—	34·4	—	35·6	—	47·0
0·2	40·8	37·7	—	35·2	—	53·0	—
0·3	—	—	50·9	—	45·4	—	65·1
0·4	52·2	52·3	—	42·9	—	69·3	—
0·6	—	—	66·6	—	60·5	—	69·8
0·8	65·9	66·7	—	57·6	—	71·8	—
Age of serum	2 days	Fresh	Fresh	2 days	Fresh	1 day	Fresh
Temp. at beginning and end of exp. °C.	47·4–46·8	47·5–46·9	47·2–47·4	47·0–46·8	47·2–47·4	47·4	47·2–47·4

Fig. 1 gives the graphical representations of these numbers; from which it will be seen that the activity for small doses of serum, whether the same individual or different animals of the same species be studied, represents a more or less definite degree of activity—there being but little separation between the individual curves in this region. For larger doses, the curves tend to separate, suggestive perhaps that the enzyme content of the blood is not absolutely fixed, but that it varies at times within certain limits. For the individual, however, the enzyme content of the blood does appear to be remarkably fixed. This is well brought out in the case of the dog 4–*P* (Fig. 1), the activity curves of which relate to two specimens collected at an interval of 1 week, the two curves being practically identical. The same thing is also seen, though perhaps to a less marked degree, in connection with the dogs 2–*P* and 3–*P*. In regard to 2–*P*, an interval of 1 week separated the specimens 1 and 2, and an interval of 2 weeks the specimens 2 and 3; while for 3–*P* an interval of 44 days separated the specimens 1 and 2.

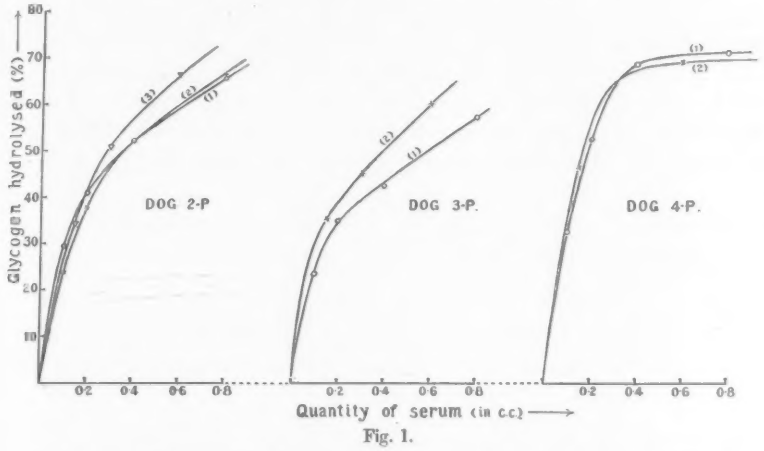

Fig. 1.

Horse. The figures with two different specimens of horse serum, both utilised 4 days after collection are set out in Table II.

Table II.

Dose of serum employed	Glycogen hydrolysed %	
	1	2
0·4	—	1·2
0·8	1·8	2·6
Temp. of exp.	47·4–47·2	46·0–45·9

On graphical representation these figures give the curve represented in Fig. 2, which proves the glycogenolytic power of horse's serum to be extremely feeble.

Ox. Only one specimen of ox serum was examined. This was used 2 days after collection, the temperature during the action being 45·5–45·7°. The results are shown in the curve in Fig. 2.

Pig. One specimen only of pig's serum was examined, the serum at the time of testing being 15 days old; temperature 46°. See the corresponding curve in Fig. 2.

Sheep. A specimen of sheep's serum, shed 26 days, and preserved sterile in presence of toluene, was tested for glycogenolytic power at a temperature of 46·5°. See the curve in Fig. 2.

Blood Maltase Negative Group.

Under this heading, sera from the cat, the monkey, the rabbit, and man were investigated.

Cat. Only one specimen was examined, the serum used being 4 days old; temperature 46·4–44·5°. See Fig. 2.

Monkey. This examination relates to a specimen of blood obtained from a *Macacus cynomolgus*. The serum was fresh when used, that is to say, as soon as clotting began the serum was at once separated from the clot by centrifugation, and the activity experiment then put up. The specimen was without any action on maltose in doses of 0·2 and 0·4 cc. in an action of 16 hours' duration at 46°, thereby proving, as was to be anticipated, that the monkey belongs to the blood maltase negative group of mammals. See Fig. 2.

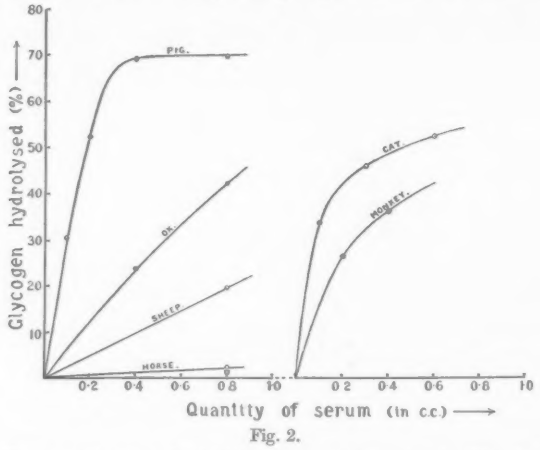

Fig. 2.

Rabbit. Four specimens of rabbit's sera were examined. Blood was collected from 2 rabbits at a time, the blood being "pooled," in connection with specimens 1, 2 and 3, so as to obtain sufficient serum for these and other experiments undertaken with it. The 4 specimens, therefore, correspond in all to 7 rabbits. Table III gives the numbers obtained, and the respective curves are indicated in Fig. 3.

Table III.

Dose of serum employed	Glycogen hydrolysed %			
	1	2	3	4
0·1	8·0	—	—	14·4
0·2	16·9	—	—	—
0·3	—	—	24·4	—
0·4	—	31·0	—	—
0·5	33·6	—	—	37·5
0·8	—	41·0	—	—
1·0	—	—	—	55·0
Age of serum	1 day	1 day	4 days	Fresh
Temp. of exp. °C.	47	46–47	47	46·0–47·4

Man. The activity of normal human serum in glycogenolytic power was tested on three occasions as follows: (1) with a single specimen of serum; (2) with a "pooled" specimen of seven negative Wasserman sera; and (3) with a "pooled" specimen of eight negative Wasserman sera. The figures obtained are given in Table IV.

Table IV.

Dose of serum employed	Glycogen hydrolysed %		
	1	2	3
0·4	4·4	8·0	15·5
0·8	20·8	20·8	25·8
Age of specimen	1 day	6 days	3 days
Temp. of exp. °C.	47·2	47·2	45·6–45·7

When these numbers are represented graphically, the two curves of the right-hand portion of Fig. 3 are obtained. There is the suggestion of a period of lag portrayed by the curve relating to specimens 1 and 2. Whether such findings are accidental, or real, will require further investigation to settle.

Discussion of Results.

From a consideration of Fig. 1, and the first section of Fig. 2, it will be seen that positive blood maltase mammals, at least the limited number studied by us, when classified in terms of decreasing glycogenolytic activity of their blood give rise to a series, as follows: pig, dog, ox, sheep, horse.

This series presents a slightly different arrangement to that of the corresponding series representing decreasing activity in *maltase*, the latter, under the same experimental conditions being: pig, dog, goat, sheep, horse and ox [Compton, 1921]. A noteworthy point of contrast here, which merits attention, is the comparative richness of the serum of the horse in *maltase*, as compared with its extremely feeble *glycogenase* content. Perhaps, indeed, it would be more in accordance with the real facts, in this connection, to speak of glycogenolytic power; because, however feeble this may be, increased activity can always be brought out by suitable p_H alterations of the medium.

Do the foregoing series of sera, of diminishing enzyme content, correspond to any other known properties of sera? Among possible properties one thinks of toxicity. It is a well-known fact that the blood of one animal when transfused into an animal of a different species, is often very toxic: a toxicity which in

the first instance was attributed to the corpuscles. But it has been proved, to quote Richet [1921], that "the real toxic element is the serum itself." It may then be asked, if the serum of one animal contains substances which are toxic for an animal of another species, may not the differing enzyme content of sera play a rôle in this phenomenon? As a matter of fact the toxicity of various sera for man, on intravenous or subcutaneous injection, is given by Richet [1921], in terms of diminishing toxicity, as: dog, goat, ass, horse. Horse serum, then, is the least toxic of mammalian sera for man; and we have shown that, of all the sera studied by us, it possesses the feeblest glycogenolytic power.

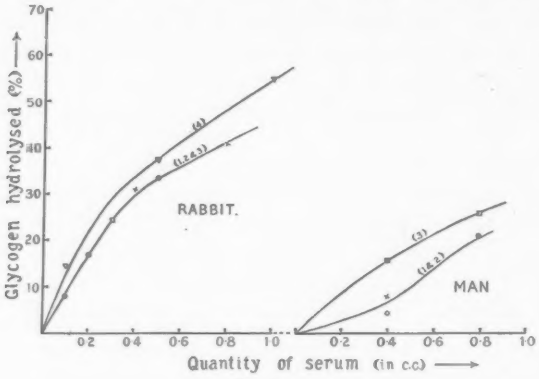

Fig. 3.

But, this conception, of serum toxicity being a function of enzyme content, will be found to rest already on solid ground, if examined from quite a different aspect: the action of heat upon the toxicity of serum. In the case of the most toxic sera known, namely those of the eel—whose serum kills in the dose of $\frac{1}{20}$th of a cc. per kilogram—the lamprey, certain vipers and cobras, whose serums are almost as toxic as their venoms, Richet states that when heated to 58°, or even lower, the toxic properties of such sera are mostly abolished; so much is this so, that the use of heated sera to-day tends to become general. The progressively destructive action of heat on any solution of enzymes hardly requires being insisted upon.

When the mammals of the negative blood maltase group are studied, it is seen from Fig. 2 (right-hand section) and Fig. 3 that they arrange themselves, in terms of decreasing glycogenolytic activity of their blood, as follows: cat, monkey, rabbit, man.

It is of some interest that man, in this group, should be lowest in the scale of blood glycogenolytic function. Of course that may simply be accidental, and further studies may reveal negative blood maltase mammals of still lower glycogenolytic power. But if not, then it points to the possible fact—allowing for exceptions, like the rabbit above—that loss of glycogen hydrolysing power of blood occurs as we ascend in the mammalian scale.

I would mention here, in passing, that Twort and Archer [1923] have found a practically identical arrangement for animals of our negative blood maltase group, so far as the content of their blood in *lipase* (monobutyrinase) is concerned. In terms of decreasing *monobutyrinase* content the negative blood maltase mammals studied by these workers arrange themselves in the following series: guinea-pig, cat, rabbit, man.

But it may be asked to what purpose are enzymes met with in blood? Some authorities, like Carlson and Luckhardt [1908] consider that they serve no essential end in the body processes, that they are mere "discards" of the tissues on the road to destruction or elimination, and that such differences as are noticeable, for instance in the amylase of blood, at different times appear to be of an accidental nature; or again, as stated by Moeckel and Rost [1910], that they play no rôle in the economy, that oscillations in the amylase of blood are merely expressive of variations in the digestive processes in some organ of the body.

I find it difficult to subscribe to the views thus expressed, that blood enzymes play no rôle in the economy, or that they are simply waste products on their way to elimination and destruction. That these enzymes in the blood are functionally active, and that they are active to different—but more or less definite—degrees for different mammalian species, is at least suggestive that they are present in the blood for their own specific purposes. As shown particularly for the individual dog, successive examinations, under as nearly as possible constant experimental conditions, reveal a remarkable constancy of enzymic activity. For the individual animal, the glycogenolytic activity of the serum gives one the impression of bearing the stamp, so to speak, of the individual; the activity curve is, in this intimate way, perhaps more characteristic of the individual than it is even of the species. In other words, the individual stands portrayed in the enzymic activities of his blood.

It is a pleasure here to express my special indebtedness to M. Albert Frouin who kindly supplied me with the specimens of dog's blood for the investigation, also to Dr C. Levaditi who furnished me with the blood of the monkey; while my thanks are due to Mlle L. Castlenau for help with some of the sugar estimations.

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LXVII. THE ACID NATURE OF OXYHAEMOGLOBIN.

By ARCHIBALD VIVIAN HILL.

From the Physiological Laboratory, Manchester.

(*Received June 22nd, 1923.*)

The direct demonstration that oxygenated blood is more acid than reduced blood is a difficult experimental feat, owing to the small change of c_H produced in blood by saturation with oxygen. Indeed Bayliss [1923] throws some doubt on the phenomenon, saying that "the chief evidence rests on measurements with the hydrogen electrode and the differences found are within the errors said by Sörensen to be inherent in the method when proteins are present." As a matter of fact the difference found by Parsons [1917] was indeed small, but perfectly definite, and the obvious accuracy and care with which his observations were made scarcely merit the criticism that they lie within the limits of error of the method. The phenomenon is not so unimportant as Parsons' small observed difference might lead one casually to suppose; the very efficient buffers present in the blood naturally lower, to a considerable degree, the rise of c_H due to the production of a small quantity of a stronger acid, and it is not fair to regard the small observed difference of c_H as the chief evidence for the phenomenon. The known effect of acid upon the oxygen-dissociation curve makes it *inevitable* thermodynamically that there should be a corresponding action of oxygen upon the c_H of blood: and the known effect of CO_2 on the same dissociation curve makes it similarly inevitable that there should be a corresponding action of oxygen upon the combination of CO_2. The effects of CO_2 on the dissociation curve are, as shown by Barcroft and Murray [1923], entirely due to the change of c_H produced by the CO_2: so that, conversely, the effects of oxygen on the CO_2-combination are entirely due to acid liberation effected by oxygenation. Hence we have three well-known effects, those of acid and of CO_2 on the oxygen dissociation curve, and that of oxygen on the CO_2 combination, all of which—as facts—are entirely beyond dispute, and each of which is a certain proof of the enhanced acidic properties of haemoglobin on combination with oxygen.

According to Henderson [1908, 1909, 1920], Hasselbalch [1916], Parsons [1919], van Slyke [1921], and many other workers, the whole of the carbon dioxide carried in blood, otherwise than in solution as CO_2, is there as bicarbonate ions. Hence the effect observed by Christiansen, Douglas and Haldane [1914], viz. the reduction in the combined CO_2 resulting from oxygenation, must be

attributed to the formation of some fixed acid about as strong as CO_2: the diminished CO_2 capacity is then due to the combination of the available alkali with this other acid. In Fig. 1 the original observations of Christiansen, Douglas and Haldane, are shown, and the upper line through the observations on the reduced blood of J. S. H. is their curve. Let us assume that the combination with oxygen has liberated fixed acid, comparable in strength with CO_2, in amount equivalent to AB (see Fig. 1). Join A to the origin O, and draw BC horizontally to meet OA in C. Then C in the diagram represents a point at the same c_H as A, since the ratio $p.CO_2/v.CO_2$ is the same both for A and C, and it is known that in blood—over a fairly wide range of CO_2 pressures—c_H

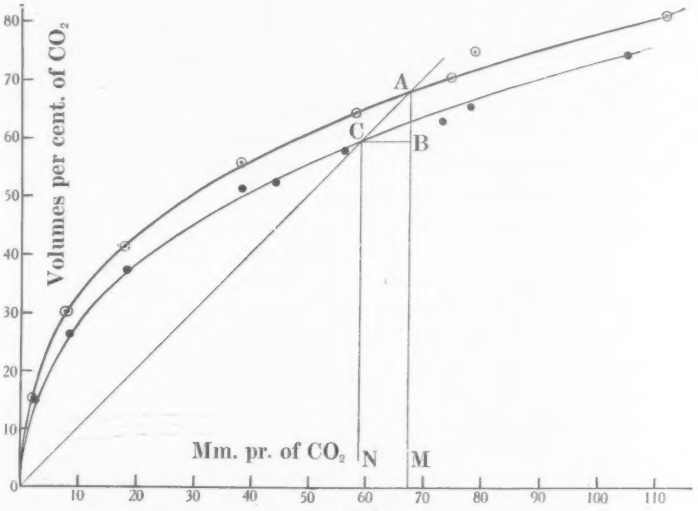

Fig. 1

is proportional to $p.CO_2/v.CO_2$. Hence at C any acid in the blood is dissociated to the same degree as at A, so that the same amount of alkali is available at C as at A to combine with added acid or with CO_2. Thus C is a point on the dissociation curve of the blood after a small amount of fixed acid AB has been added to it. By shifting A along the upper curve, and repeating the construction, the whole of the lower curve can be reproduced. In Fig. 1, AB is taken as 8·4 cc. of CO_2 per 100 cc. of blood, and it is seen that the lower curve drawn in this way fits the observed points with complete satisfaction. Thus the effect of oxygen upon the CO_2 dissociation curve of the blood of J. S. H. may be expressed by the statement that the change from complete reduction to full oxygenation has simply liberated fixed acid, of strength comparable with CO_2, in amount equivalent (as acid) to 8·4 cc. of CO_2.

According to the theory put forward and tested by Brown and the present writer [1923] the combination of oxygen with haemoglobin, proceeding accord-

ing to the scheme $H(Hb)_n + nO_2 \rightleftarrows H(HbO_2)_n$ leads to the formation of the stronger acid oxyhaemoglobin which dissociates according to the scheme, $H(HbO_2)_n \rightleftarrows H^{\cdot} + (HbO_2)'_{n'}$. From experimental data provided by Barcroft and Murray [1922] the acid dissociation constant of oxyhaemoglobin was calculated and found to be about equal to that of H_2CO_3. According to this theory the stronger acid formed, viz. one molecule of $H(HbO_2)_n$, which like CO_2 must be almost completely dissociated at the c_H of blood, is equivalent in amount to $1/n$th of the oxygen taken up. This is simply tested. The most probable value of n for blood was found by Brown and Hill, from other considerations, to be about 2·2. Assuming the blood of J. S. H. to have held the normal amount of 18·5 vols. of O_2 %, $1/n$th of this is 8·4 cc. This is precisely the amount by which it is found necessary to lower (at constant c_H) the curve for reduced blood, in order to make it fit the observations on oxygenated blood. No more striking testimony could be demanded to the general adequacy of the theory, than (*a*) the accuracy with which the curve for oxygenated blood can be deduced from that for reduced blood, and (*b*) the precise agreement of the single constant (the height *AB*) necessary for that deduction with the quantity required by the theory.

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LXVIII. NOTE ON THE COLOUR CHANGES IN RATS' FUR PRODUCED BY ALTERATIONS IN DIET.

By GLADYS ANNIE HARTWELL.

From the Physiological Laboratory, King's College for Women, Household and Social Science Department.

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The observations described in this paper arose incidentally in the course of other work and it is thought desirable to pubish a brief account, inasmuch as the results may be of interest to others who are working definitely along such lines.

Niemes and Wacker [1922] found that grey-black rats turned brown when fed for a long period on a diet of skim milk and starch; further, the animals regained their original colour when put on a normal diet. Rats fed on starch and full-milk showed no change in colour. These observers suggest that, although the hair-pigment is usually assumed to be derived from protein products (*e.g.* tryptophan, tyrosine), it is possible that other substances are also needed for the manufacture of the pigments, and they put forward the suggestion that the absence of cholesterol from the diet is responsible for the colour change they noted.

Garrod [1909] expresses the view that "the trend of opinion at the present day is towards the view that they (*i.e.* the melanins) are derived from proteins in general, perhaps by the action of a tyrosinase, and that the natural melanins are allied to the melanoid substances which are formed during the hydrolysis of proteins."

The recent work of Saccardi [1922, 1, 2] suggests a pyrrole ring structure for melanins with the possibility of adrenaline as a precursor.

The experiments to be described here indicate a relation between the dietary protein and pigment formation. During the summer months of 1920 some control pied-rats fed on bread and whole milk, began to lose their brown-black colour and became a grey-fawn. About three weeks after the first difference was noticed, the animals had no black hairs at all, and were entirely white and a grey-fawn. It was hot weather at the time and hence it was thought that some preservative in the milk might be responsible for the change in colour. Accordingly other experiments were started using milk from two separate sources, and in 2–3 weeks all the rats showed some change in colour and eventually all the animals became light-coloured. They remained thus for a time, but eventually their hair darkened again, although it was never really black.

Similar results were obtained again in the following summer, but no change of colour could be produced in the winter when the animals were growing more slowly. In 1922 this change of coat colour was obtained in 60 growing rats, some fed on bread and whole milk alone, and others on bread and milk supplemented with kitchen scraps (largely vegetable). Other experimental animals on a high protein diet had jet black coats and showed no variation in colour; therefore it seemed that addition of extra protein might lead to a darkening of the coat.

Twenty-four of the light-coloured animals were divided into two groups; one lot were kept on bread and milk (30 g. bread to 100 cc. milk), and the other half had bread and milk and caseinogen (30 g. bread, 100 cc. milk, 9 g. "food casein" [see Hartwell, 1922]). The latter showed a distinct darkening of their coats at the end of the first week; the coloured patches began to darken at the edges and the darkening spread inwards until at the end of the third week the animals were black and white. The jet blackness of the black was noticeable.

Another series of light-coloured animals were put on a high protein diet consisting of 15 g. bread, 6 g. "food casein," 1·5 g. butter, 0·7 g. salt mixture [Hartwell, 1922], 50 cc. of 3 % marmite. These showed changes exactly similar to those observed in the rats fed on bread and milk and caseinogen. In both cases the rats' coats became black, when the animals were fed on large quantities of protein rich in tyrosine and tryptophan. These experiments, therefore, support the view that the melanins are formed from tyrosine and tryptophan.

Other points noted were:

1. The fur is very thick when the animal is fed on a high protein diet.
2. Adult rats rarely show any change in coat colour, but a few instances have been noted—for example, when the stock animals were fed on kitchen scraps poor in protein, a few animals became lighter in colour. (Just over 1000 litters have been experimented with and only one suckling rat had a light coat, which was noticed about the eighteenth day of suckling.)
3. Microscopically the light-coloured hairs appear similar to the dark ones, except that the pigment is much lighter in shade.

I wish to thank Prof. V. H. Mottram for his help in this work, the cost of which was defrayed by a grant from the Medical Research Council.

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LXIX. A HEAT-STABLE CATALYST IN ANIMAL TISSUES WHICH DESTROYS THE IMINAZOLE RING AND UNMASKS AMINO GROUPS.

By WINIFRED MARY CLIFFORD.

From the Physiological Laboratory, Household and Social Science Department, King's College for Women, Kensington.

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An account of the catalytic destruction of carnosine *in vitro* at 100° by a substance present in skeletal muscle and liver has been previously published [Clifford, 1922]. These experiments showed a progressive loss of the iminazole ring when a solution containing carnosine was kept with skeletal muscle or liver over a period of 3–4 weeks. The amount of iminazole was estimated by a colorimetric method described previously [Clifford, 1921, 1], depending on the coupling of the histidine molecule of carnosine with diazo-reagent. The loss of red colour could be accounted for in two ways. The ring substance might have been joined to others in a more complicated manner, similar to that of the histidine in proteins, and thus be unable to couple with the reagent. Another and more probable solution was that the catalyst actually broke up the ring into some simpler substance. In the latter case it was possible that the nitrogen of the ring would be converted to amino nitrogen and then the increase of amino nitrogen could be estimated by van Slyke's method. Accordingly experiments were carried out in which parallel estimations of diazotisable iminazole and amino nitrogen were made.

Iminazole decrease and Amino N increase in Beef and Water at 100°.

3–5 g. of finely minced beef were placed in test-tubes with about 15 cc. water and the tubes were plugged with cotton-wool and left in a constantly boiling water-bath for 17–24 days. Water was added to the test-tubes when necessary to make up for that lost by evaporation.

At intervals a tube was removed, about 30 cc. water and 5 cc. of 20 % metaphosphoric acid added and the whole made up to 50 cc. and left to stand 12–24 hours. After filtration part of the protein-free solution was tested colorimetrically for carnosine [Clifford, 1921, 1] and van Slyke estimations for amino acids were performed on the other portion, the micro-form of the apparatus being used.

The results are shown in Fig. 1, Curves 32 and 41.

From these results it can be seen that in both cases a three-stepped curve was obtained but in opposite directions, *i.e.* a fall of iminazole was accompanied by a rise of amino acid in the protein-free extract. The rise of amino N is, however, above that which could come from carnosine in the solution and

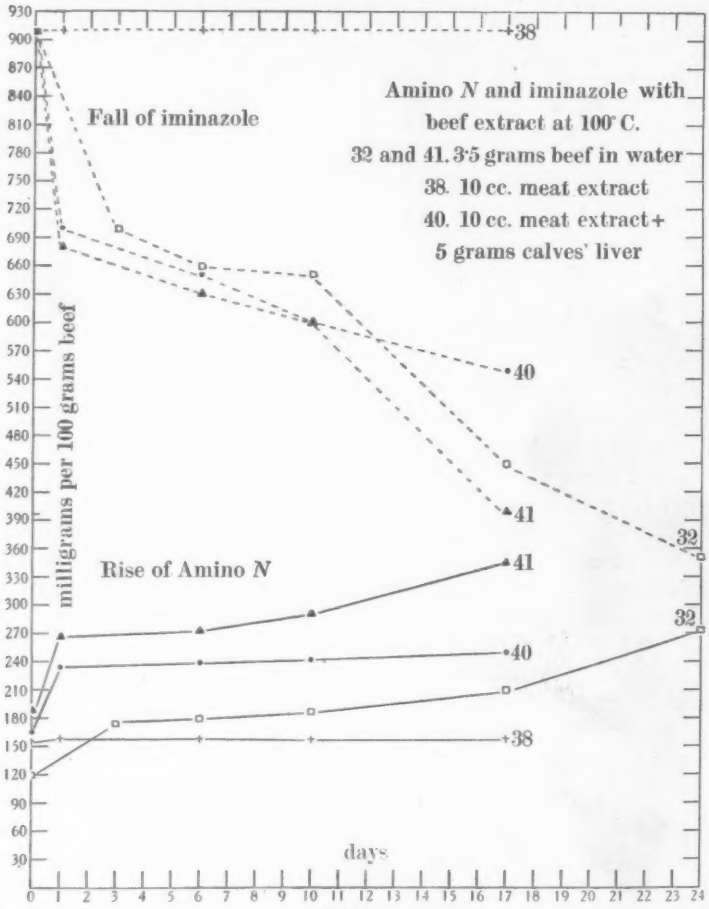

Fig. 1.

thus must come from a hydrolysis of the beef protein itself, or from some nitrogen-containing extractives. These substances are affected in a similar way to the iminazole ring of carnosine and also give a rise of amino N in the same three-stepped manner.

That there are considerable amounts of other soluble extractives giving amino N can be seen from the first figures of Expt. 32 where with meat extract containing carnosine to the extent of 910 mg. per 100 g. meat, 119 mg. amino N per 100 g. were present in place of 52 mg. if the whole came from carnosine. In the case of Expt. 41 again there are 187 mg. per 100 g. instead of 48 mg. if due entirely to carnosine.

The fall of carnosine and rise of amino N is against the findings of Baumann and Ingvaldsen [1918] who found muscle and liver extract to be without any hydrolytic action on carnosine. Their experiments, however, assuming enzyme action, were carried out at 40° for a period of 24 hours only.

In one of the author's experiments in which meat and water were sterilised by boiling for 30 minutes and then left at 37° no change in carnosine content was observed in 13 days, so it is unlikely that at 40° any change should take place in 24 hours.

No rise in amino N with Beef Extract.

It was thought possible from the above experiment that the rise in amino N might be due to hydrolysis of soluble protein in the extract, though seven previous experiments [Clifford, 1922] showed that in an extract kept for 13–16 days no change in carnosine content occurs.

Therefore 10 cc. of an extract made by boiling 60 g. of beef in two separate portions of 150 cc. water were placed in each of a series of test-tubes, plugged and left at 100°.

Estimations of carnosine and amino N were carried out on these at intervals. The carnosine remained constant, and the amino N showed an insignificant rise of 4 mg. per 100 g. meat extracted, whilst in the previous experiment the rise was 158 mg. per 100 g. (Fig. 1, 38.)

It is possible that the very small protein content of these tubes compared with that of those in which 5 g. of meat including its fibrin remained in a small bulk of water could account for the difference. This is improbable but experiments with strong protein solution in water at 100° are in progress to ascertain if a large rise of amino N in a three-stepped curve will result.

The heat-stable catalyst responsible for the results obtained above is not confined to muscle tissue as the following experiments will show.

Rise of amino N and Decrease of Iminazole with Liver in Meat Extract.

To separate test-tubes, each containing 10 cc. of beef extract made by boiling minced beef with water and filtering, 5 g. of calf liver were added. These tubes were plugged and placed in the water-bath and treated in the same way as the beef and water tubes. Again there was a rise in amino N and a fall in carnosine content, both with the usual curve. The rise of amino N once more was greater than could be accounted for by the conversion of the diazotisable N to amino N (Fig. 1, 40), indicating the presence of other substances than carnosine, which are split by the same agent in a similar way to carnosine.

The question then arose as to whether the catalyst would destroy iminazole compounds other than carnosine. Histidine was therefore used in place of muscle extract and the following experiments were carried out.

Effect of Cod Muscle on Histidine Solution.

Cod muscle has previously been shown to be free from any iminazole substance capable of coupling with diazo-reagent [Clifford, 1921, 2]. It has also been shown to contain a catalyst which destroys carnosine [Clifford, 1922], and was therefore used to determine if the catalyst could act on a simpler iminazole compound.

Separate tubes, containing 10 cc. of 0·05 % histidine monohydrochloride solution, were taken and 5 g. of minced cod muscle were added. These tubes were treated in the same way as those with beef and water. Again the iminazole which could be diazotised was destroyed, and there was a rise of amino N. This was greater than could have come from the histidine and therefore must have come from the cod. At the end of 21 days there was a loss of histidine which could account for a rise of 9·9 mg. of nitrogen if all the ring N were converted to NH_2, whilst the actual rise was 43 mg.

However, the shape of the curves was the same as with carnosine in meat extract, both with regard to iminazole fall and to amino N rise (Fig. 2, 35). From this it would appear that the catalyst will attack histidine in the same way that it will attack β-alanyl-histidine and may be capable of splitting any iminazole ring.

Effect of Liver on Histidine Solution.

The experiment was carried out as for cod, except that 5 g. of minced calf's liver was used in place of cod muscle, again with the idea of introducing a tissue containing the catalyst which at the same time was devoid of free iminazole. The result was similar to that obtained with cod, namely, a fall of iminazole giving a red colour when diazotised, and a large rise of amino N (Fig. 2, 36).

The rise of amino N here is 42 mg. whilst the most which could have come from the destroyed histidine is 8 mg.

Effect of Washed Meat on Histidine Solution.

It has previously been shown [Clifford, 1922] that meat which has been minced and washed in running water for 24 hours still contains the catalyst, though obviously devoid of all water-soluble substances. Accordingly 5 g. of minced beef washed for 24 hours were added to another series of tubes containing 10 cc. of 0·05 % histidine monohydrochloride solution. On repeating estimations as for the cod muscle and calf liver series similar results were obtained. There was a still greater rise of amino N, it being 126 mg. at the end of 21 days against a possible 9·2 mg. from histidine nitrogen (Fig. 2, 37).

That the destruction of the histidine ring is not mere hydrolysis at 100° is shown by the following experiment.

Effect of Temperature on Histidine Solution.

Tubes containing 10 cc. of 0·05 % histidine monohydrochloride solution were placed in the bath at 100° and used as controls. In 21 days there was no alteration either in iminazole content as estimated colorimetrically or in amino N as estimated by the method of van Slyke.

0–3–5–9–17–21 days; histidine 50 mg.; amino N 3·7 mg. (Fig. 2, 34).

From this experiment it appears that there is no spontaneous splitting of the histidine ring when a weak solution is kept at 100° constantly for 3 weeks.

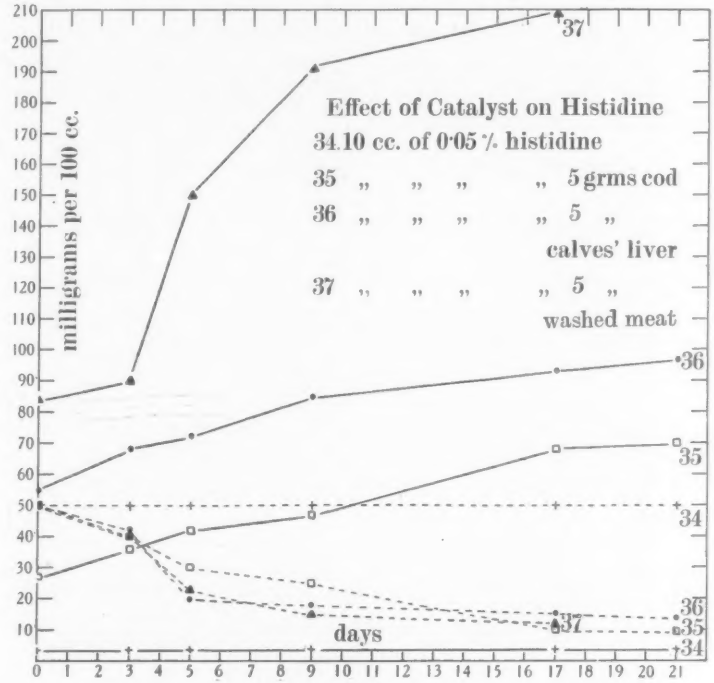
Effect of Catalyst on Histidine
34 10 cc. of 0·05 % histidine
35 „ „ „ „ 5 grms cod
36 „ „ „ „ 5 „ calves' liver
37 „ „ „ „ 5 „ washed meat
milligrams per 100 cc.
days
37
36
35
34

Fig. 2.

Discussion of Results.

The catalytic agent present in beef and cod muscle and in liver is capable of destroying carnosine in a beef extract, and also of destroying histidine in watery solution. This is shown by a decrease of colour on diazotising histidine solution or muscle extract at intervals after the addition of cod, washed beef or liver, the tubes being kept at 100°. This may indicate the existence of a catalyst capable of splitting any iminazole ring.

The peculiar shape of the curve previously observed in the disappearance of carnosine is maintained with histidine solution. At the same time that there is a fall in iminazole there is a rise in amino N which gives a curve in the opposite direction to the iminazole fall. This suggests an opening of the iminazole ring with the changing of its ring nitrogen to amino N.

The rise of amino N is greater than can be accounted for by the opening of the diazotisable iminazole ring and therefore it must in part come from some other source. The protein of the added substance (muscle or liver) may be hydrolysed with the consequent freeing of amino groups. This is improbable as a watery meat extract which necessarily contains some protein can be kept 17 days with a rise of only 4 mg. per 100 g. extracted meat and the iminazole content remains unchanged. Another objection to the simple hydrolysis of protein as a cause of the rise of amino N is the peculiar shape of the curve obtained. This is the converse of that of loss of diazotisable iminazole and suggests a connection between the two changes. This is shown well in Fig. 3, Expt. 35, where the scale has been enlarged.

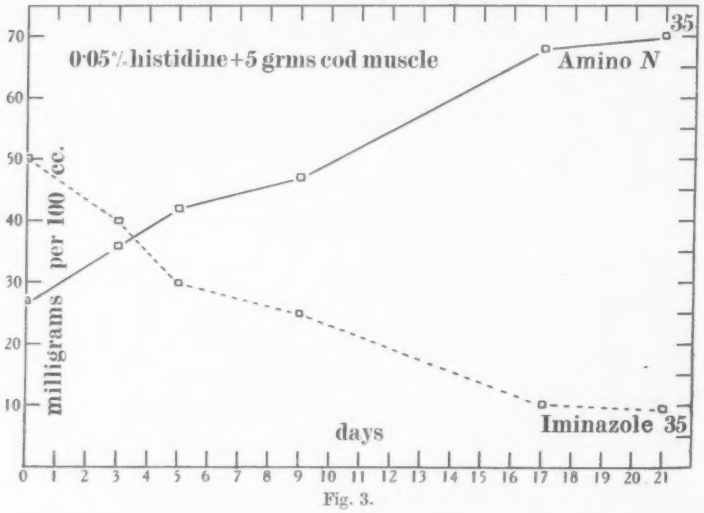

Fig. 3.

The catalyst may be able to attack other iminazole compounds, so that other extractives besides carnosine may give rise to the extra amino N. There is a possibility that purine bodies such as xanthine and hypoxanthine may have their iminazole group split off as urea, and this could account for discrepancies between ingested purine and excreted uric acid in the body. Creatine is, through guanidine, allied to arginine, and also is known to give rise to creatinine spontaneously when boiled. Creatinine shows the iminazole ring formation and therefore is allied to histidine and the purines. The breaking

of the creatinine ring may be possible by the action of the catalyst destroying histidine, when creatine might result. This would give possibilities of a link between arginine and histidine and their interchangeability in the body.

Experiments are in progress in order to enquire into these various possibilities.

Thanks are due to Professor V. H. Mottram for his advice and interest in the work.

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LXX. POLARIMETRIC OBSERVATIONS ON SOLUTIONS OF GLUCOSE SUBJECTED TO CONTACT WITH INTESTINAL MUCOSA OF RABBIT.

By DAVID STIVEN and EDWARD WAYMOUTH REID.

From the Physiological Laboratory of University College, Dundee.

(*Received July 4th, 1923.*)

In a paper by Hewitt and Pryde [1920] published in this Journal, three polarimetric experiments with previously equilibrated hypotonic solutions of glucose, which had been in contact with the mucosa of the small intestine of the rabbit for five minutes, are quoted with data which are claimed as indicative of a disturbance of the equilibrium of the solutions as a result of the intestinal contact.

An upward mutarotation of the solution in the polarimeter to the $\alpha - \beta$ equilibrium value is quoted for each of the three cases, the conclusion drawn being that a downward mutarotation had taken place earlier, *i.e.* during the period of contact.

The polarimetric observations were commenced from $3\frac{1}{2}$ to 10 minutes after removal of the solution from the gut, and the mutarotation effect had ceased to be evident from 15 to 25 minutes after removal.

The observers, however, state on pp. 399–400 of their paper that in some experiments these mutarotation effects were "ill-defined," and in some "completely negative," but the relative numbers of the experiments yielding the three types of results are not given.

As, so far as we are aware, the positive result of these observers has not been controlled, we have thought it advisable to repeat the experiment. One of us (D. S.) is responsible for the polarimetric and analytical work, and the other (E. W. R.) for the operative procedure.

The observer with the polarimeter took no part in the operation upon the animal, and entered a dark room ten minutes before taking an observation, for the double purpose of attaining a fair degree of dark adaptation of the eye for the earliest readings, and the necessary mental composure.

Since the question as to whether or no any mutarotation occurs in the glucose solution during the early period after its removal from the gut is, in this particular case, of more importance than quantitative values in relation to the concentrations of glucose given by analyses, no attempt at complete chemical removal of the small amounts of protein necessarily added to the solution from the intestinal glands during its sojourn in the gut, was attempted.

In cases such as that before us, we are, moreover, of opinion that chemical treatments may hold an element of danger.

Barring the necessary filtration, the only treatment to which we have ever subjected the solution has been to shake it, previous to filtration with a suspension of aluminium hydroxide. The p_H of the liquid of this suspension was 7·3 by the indicator method, and it was considerably less alkaline, by the same test, than the glucose solution as removed from the gut. The positively charged particles of this suspension are remarkably efficient in clarification, and the filtrate of gut contents so treated may give no "ring" with Tanret's reagent, and in all cases it is perceptible with difficulty.

In many experiments, as evident in the tables, we have purposely omitted the alumina treatment, but though such omission often entailed resort to a more powerful light source than that of sodium flame, it was found to be absolutely without effect on the order of the polarimetric readings, provided that the filtrate was clear enough for good readings.

Procedure. Rabbits, starved 24 hours, anaesthetised by ether, and tracheotomised were employed. About 30 inches (live length) of small intestine, which had been washed free of debris by Ringer-Tyrode solution at 38°, were used, and from 20 to 30 cc. of glucose solution at 38° were run in, distention of the gut being avoided.

No loss of epithelium ever occurred, and, during the contact period, cloths, wrung out in warm saline solution, protected any part of the gut which was not enclosed within the abdominal cavity.

Usually the solution was allowed to lie in contact with the mucosa for five minutes, and if peristalsis was not marked, an occasional gentle manipulation was employed to bring fresh parts of the contents into contact with the epithelium.

Occasionally the solution was kept in the gut for ten minutes, but no difference was noted, so far as mutarotation is concerned, in such instances. The time of removal of the solution from the gut was "clocked," and the clock handed in to the observer in the dark room, as soon as the filtration and filling of the polarimeter tube were completed.

Filtration. Since clarity is a *sine qua non* in a solution, the polarimetry of which is to be reliable, particular attention was devoted to this matter. Clarity of filtrate has, however, in this matter, to be combined with speed of filtration, and the latter condition rules out many otherwise excellent methods. The Balston "filter-hat" used by Hewitt and Pryde is quick, but we found it often at fault in the matter of clarity of filtrate.

We have used a pair of Gooch crucibles, with asbestos mats, built up freshly for each experiment on a disc of filter paper, the two crucibles being in parallel on the suction system, and used contemporaneously.

The number of crucibles in parallel can, of course, be increased, but we found two sufficient for the purpose of getting enough filtrate in a short time. The suction system stood ready with a partial vacuum of about 400 mm. of

mercury, the filter-flasks being clamped off. Once the crucibles were filled with solution from the gut, release of the clamps at once set going the filtration.

In the filter-flasks were set graduated tubes to receive the filtrate, and, by this device, the moment at which enough filtrate had collected to fill a polarimeter tube was known, and no time was lost in unnecessary prolongation of the filtering process.

The polarimeter tube, filled with filtrate, and the clock giving the time at which the original solution left the gut, were at once handed in to the observer in the dark room.

The filtrates, when alumina cream is used, are "glass clear," and the Tanret protein test is negative or only just perceptible. As the glucose analyses were made on the contents of the polarimeter tubes, the slight dilution due to the use of the alumina cream was of no moment in the matter of comparison of direct and calculated readings.

We do not, however, pretend that all laevo-rotatory substances of intestinal origin added to the original glucose solution are removed by this elementary procedure. With good working asbestos mats we often got a first reading on the polarimeter in two minutes after removal of the solution from the gut, but the time which must elapse to collect a sufficient filtrate is necessarily variable, by the "build" of the mat, and the amount of the secretion which has occurred in the gut affecting the viscosity of the solution submitted to filtration. The times will be found in the tables of experiments.

Polarimetry. The instrument was a Schmidt and Haensch three-field of the Lippich type, reading direct to 0·01°, and either one or two decimeter tubes were used. The zero value of the tube (water-filled) was independently taken for each experiment. To preserve, as far as possible, the dark adaptation of the observer's eye the light for scale reading was cut down to the minimum practicable.

Though sodium light served well for the filtrate which had undergone alumina cream clarification, difficulties in sharp reading were often met with when this treatment was omitted. As already noted, we have purposely omitted the alumina treatment in some cases, though it has not in any way affected the results. While a good sample of asbestos mat will give a clear enough filtrate, without any alumina clarification, others may give a filtrate too opalescent for proper observation by sodium light.

As, at the time, we had no arrangement for intense mercury-green light we had in those cases to resort to light-filters, and a 100 c.p. "Pointolite" lamp. The filters used for the light were Wratten's M. screens. $A + B + E$ in conjunction. The abandonment of monochromatic light in such cases, while affecting the comparison of calculated and observed rotations, does not, of course, affect the question under examination, viz. whether mutarotation is or is not to be observed in the solutions shortly after removal from the gut.

Glucose solutions and analytical method. Hewitt and Pryde do not state whether the solutions introduced into the gut were simple aqueous solutions

of glucose or solutions in some type of saline liquid. Most of our experiments were done with simple aqueous solutions; occasionally the glucose was dissolved in Ringer-Tyrode solution, and, in one instance, acid sodium phosphate was added to an equilibrated glucose solution before use, to try the effect of keeping the gut contents on the acid side throughout the experiment.

The concentration of the solution subjected to polarimetry was estimated by the micro-method of Shaffer and Hartmann [1921]. The limited volume of solution available for analysis necessitated the use of a reliable micro-method so that as many individual analyses as possible might be effected for a representative mean value.

As some laevo-rotatory material is, by the nature of the experiment, always present in the mixture of glucose and intestinal secretion subjected to polarimetry, the rotation calculated from the analysis is likely to be greater than that observed. In nine cases out of the thirteen, where observations were made by sodium light, this has been found to be the case. In the experiments where the protein tests were most distinct, this difference between calculated and observed rotations was greatest.

Furthermore, in such cases the opalescence of the solutions was sometimes sufficient to impair the reliability of the polarimetric readings.

An instance, not included in the tables, is given of a series of readings from 4 to 25 minutes after removal of the solution from the gut, in which the "switch-back" character of the readings, due to absence of clarity, makes the observation valueless.

Minutes since removal from gut	4	5	6	7	8	9	10	11	12	13	14
Observed dextro-rotation in degrees	·85	·82	·80	·80	·80	·79	·78	·79	·80	·79	·77

	15	16	17	18	19	20	21	22	23	24	25
	·78	·78	·76	·76	·75	·77	·77	·80	·80	·80	·77

The erratic up-and-down variation of the readings in this instance, beyond the limits of the error of reading on a clear tube, is obviously due to the absence of certainty in such a case in adjustment of the field of the instrument. Any results of this type have been rejected.

Effects of alkalisation of contents of polarimeter tube. The addition of a drop of alkali to the contents of the polarimeter tubes, results, with one or two exceptions, in a distinct diminution of the dextro-rotation. We found, invariably, that the greater the amount of protein in the filtrate, the greater the diminution on addition of alkali, and, the clearer the filtrate, the smaller the effect of alkali.

We have also observed that the addition of weak alkali (2 % NaOH) results in no change or in a slight increase. On the other hand, the addition of a drop of strong alkali (40 % NaOH) results in a diminution sometimes of 0·1° or more in a 1-decimeter tube. These filtrates were alkaline enough, from added

Table I. *Sodium Light Readings.*

Dextrose concentration introduced into gut. Treatment of filtrate etc.	Mean dextro-rotations in degrees at minute intervals after removal from gut. Readings from 2—25 minutes																								Dextro-rotation in degrees				
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	(1) After standing one hour	(2) After adding alkali	(3) Calculated from analysis	% dextrose from analysis of tube	No.
1 % dextrose in Ringer-Tyrode. Alumina. Asbestos filters. 2 d. tube	—	·79	·78	·77	·79	·79	·81	·81	·82	·78	·79	·78	·79	·80	·78	·78	·79	·79	·79	·79	·79	·81	·79	·79	·79	·86	·82	·779	13
2 % dextrose in ·96 % NaH_2PO_4. Alumina. Asbestos filters. Hypotonic $\Delta = -0{\cdot}43°$ C. 2 d. tube	—	·45	·43	·40	40	40	·40	·41	·40	·41	·41	·39	·40	·40	·40	·39	·40	—	·40	—	—	—	—	—	·40	·37	·42	·792	18
2 % dextrose in water. Alumina. Asbestos filters. 2 d. tube	—	1·69	1·71	1·70	1·70	1·70	1·70	1·70	1·68	1·69	1·70	1·69	1·70	1·69	1·69	1·69	1·70	1·69	1·68	1·70	1·69	1·68	1·68	1·69	1·69	1·66	1·72	1·63	24
Ditto	—	—	1·39	1·38	1·36	1·36	1·36	1·35	1·36	1·36	1·34	1·35	1·34	1·36	1·37	1·34	1·36	1·35	1·35	1·36	1·35	1·37	1·36	1·35	1·35	1·30	1·38	1·31	25
Ditto	—	—	—	1·74	1·75	1·74	1·74	1·72	1·74	1·73	1·73	1·72	1·73	1·74	1·73	1·74	1·73	1·73	1·73	1·73	1·72	1·72	1·73	1·73	1·72	1·68	1·76	1·67	26
Ditto	·98	·99	·97	·96	·98	·97	·98	·98	·98	·97	·98	·98	·99	·98	·97	·97	·98	·98	·98	·98	·97	·98	·98	·98	·98	·94	1·01	1·93	28
2 % dextrose in water. No alumina. Filter hat. 1 d. tube	·79	·80	·80	·80	·78	·80	·79	·79	·80	·78	·79	·78	·80	·78	·79	·79	·80	·79	·78	·80	·80	·78	·79	·79	·79	·75	·74	1·404	11
2 % dextrose in water. No alumina. Asbestos filters. 1 d. tube	—	1·00	1·00	·99	1·01	·99	1·00	1·01	1·02	1·00	·99	·99	·99	·99	—	—	—	—	1·01	1·01	1·02	—	1·01	1·01	1·00	·98	·96	1·836	20
Ditto	—	—	·98	·99	1·02	·98	·99	1·02	1·00	1·02	1·00	1·02	·99	·99	·99	·97	·98	1·00	1·01	·99	1·01	1·00	·99	1·00	·99	·89	·99	1·88	27
Ditto	·99	1·00	1·00	·99	·99	1·00	·99	·98	·99	·99	·99	·99	1·00	—	—	—	—	—	—	·97	1·00	·99	1·00	·99	·99	·98	·99	1·88	16 (*b*)
Ditto	—	1·07	1·08	1·08	1·07	1·08	1·07	1·07	1·07	—	—	—	—	1·05	1·05	1·06	1·06	1·05	1·05	—	—	—	—	1·06	1·07	1·07	1·02	1·939	15 (*b*)
1·7 % dextrose in water. No alumina. Asbestos filters. 1 d. tube	·84	·83	·83	·81	·80	·82	·82	·83	·83	·84	·84	·82	·82	·81	·82	·84	·83	·83	·84	·82	·83	·82	·82	·83	·83	·75	·78	1·488	30
2 % dextrose in water. No alumina. Asbestos filters. 1 d. tube	—	·96	·98	·99	·98	·95	·97	·99	·97	·99	·98	·97	·99	·97	·97	·97	·98	·97	·98	·97	·96	·97	·97	·98	·98	·84	1·00	1·917	32

Table II. *Screened "Pointolite" Readings.*

Dextrose concentration introduced into gut. Treatment of filtrate etc.	Mean dextro-rotations in degrees at minute intervals after removal from gut. Readings from 3–25 minutes																						Dextro-rotations in degrees: (1) After standing one hour	(2) After adding alkali	(3) Calculated from analysis	% dextrose from analysis of tube	No.
	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25					
1 % dextrose in Ringer-Tyrode. No alumina. Filter hat. 1 d. tube	·24	·23	·23	·23	—	·24	·24	·24	·24	·24	·24	·24	·24	·24	·25	·25	·25	·24	·25	·24	·25	·25	·24	·24	·20	·21	·396	12
1·5 % dextrose in Ringer-Tyrode. Alumina. Asbestos filters. 2 d. tube	—	·53	·56	·56	—	—	·59	·58	·55	·55	·55	·55	·55	·55	·55	·55	·55	—	—	·55	·55	—	·55	·57	·56	·60	1·148	14
2 % dextrose in water. No alumina. Asbestos filters. 1 d. tube	—	—	—	·78	·79	·78	·78	·76	·76	·76	—	—	—	—	—	—	—	—	—	—	—	—	·76	·78	·67	·74	1·409	16 (*a*)
Ditto	—	—	—	·63	·62	—	·64	·64	·64	·64	·63	—	—	—	—	—	—	—	—	·64	—	·64	·63	·63	·65	·77	1·458	15 (*a*)
1 % dextrose in water. No alumina. Asbestos filters. 2 d. tube	·96	·97	·97	·97	·96	·96	·96	·96	·97	·96	·97	·96	·97	·98	·97	·98	·98	·98	·97	·97	·97	·98	·97	·96	·94	1·10	1·048	6
Ditto	1·08	1·07	1·08	1·08	1·09	1·09	1·08	1·09	1·08	1·08	1·09	1·09	1·08	1·08	1·10	1·08	1·09	1·08	1·08	1·08	1·08	1·08	1·08	1·08	·99	1·18	1·124	8
1 % dextrose in water. No alumina. Filter hat. 2 d. tube	·90	·90	·90	·89	·88	·90	·91	—	·88	·89	·87	·87	·87	·89	·89	·89	·91	·89	·88	·88	—	—	·88	·88	·80	·99	·942	9
1·8 % dextrose in water. No alumina. Asbestos filters. 1 d. tube	—	·69	·69	·70	·69	·68	·70	·70	·69	·69	·71	·69	·69	·68	·69	·69	·69	·69	·69	·69	—	·68	·67	·64	·58	·75	1·42	29
2 % dextrose in water. Alumina. Asbestos filters. 2 d. tube	—	—	—	—	—	*	1·32	1·36	1·34	1·34	1·35	1·33	1·36	1·34	1·33	1·34	1·34	1·35	1·35	1·35	1·35	1·35	1·34	1·36	1·22	1·44	1·368	3
1 % dextrose in water. Alumina. Asbestos filters. 2 d. tube	—	—	·91	·91	·91	·91	·91	·91	·92	·91	·91	·90	·91	·90	·90	·91	·91	·91	·89	—	·91	·91	·90	·91	·86	1·00	·952	4
Ditto	—	·84	·84	·83	·85	·84	—	—	·81	·81	·81	·81	·81	·81	·81	·80	·80	·81	·81	·82	·80	—	·80	·81	·74	·88	·84	5
2 % dextrose in water. No alumina. Gut washed with saline. Asbestos filters. 1 d. tube	—	—	—	·78	·78	·78	·77	·78	·78	·78	·79	·78	·78	·78	·79	·79	·79	—	—	—	·79	·79	·78	·79	·72	·87	1·65	22
2 % dextrose in water. No alumina. Asbestos filters. 1 d. tube	—	·82	·81	·80	·79	·79	·80	·79	·80	·79	·80	·80	·79	·80	·79	·79	·79	·80	·80	·79	·79	·79	·80	·80	·69	·91	1·74	31

* Clogged filter.

intestinal secretion, to turn litmus paper blue before addition of the alkali. The same drop in rotation can be got by adding hydrochloric acid. An example of the effect of weak alkali can be seen in the Expts. 13, Table I, and 15 *a*, Table II, while the others all show the effect of a drop of the strong alkali.

These observations lead us to believe that this drop in rotation is due to the protein forming salts with alkali or acid, these salts being more laevorotatory than the natural protein—an observation made, among other workers, by Pauli [1914].

Remarks.

It is evident that, so far as polarimetric evidence goes, our experiments give no support to the idea that a disturbance of equilibrium associated with the formation of a more reactive type of sugar occurs as a result of contact of a stablilised glucose solution with the mucosa of the small intestine of the rabbit.

It appears to us that, even if some reactive type were formed, it is very unlikely that evidence of its production would be obtained by the method adopted by Hewitt and Pryde.

Not only would one expect the special type of sugar formed to be at once taken up into the blood-stream, if the transformation was a necessary preliminary to absorption, and so to be removed from the solution used for experimental observation, but, even if traces of the special type still remained in the solution withdrawn from the gut, its existence in unstable form is likely to be momentary.

According to Irvine [1923] the gamma types of both glucose and fructose revert *instantaneously* to the stable form even in the presence of 0·1 % HCl.

Aqueous solutions of glucose which have been in contact with the gut wall for 5 minutes are always alkaline, so far as our experience has gone.

To prove the thesis that a reactive type is formed, some method of condensation to a stable derivative would appear to be necessary for, as Irvine remarks, "there is little prospect of detecting these fugitive isomerides by ordinary processes."

To effect such a "fixation" within the organism, preliminary to extraction and investigation, seems to be the problem before the searcher for evidence of the transitory presence of reactive types of sugar in metabolism.

Conclusion.

The experiment of Hewitt and Pryde has been repeated thirty-four times. In eight cases the solutions obtained from the gut had to be discarded as not clear enough for good polarimetry.

In the remaining twenty-six experiments in no single instance have we seen any sign of mutarotation in the earlier minutes after removal of the solution from the gut.

The rotation has remained steady within the observer's known error of adjustment of the field of the polarimeter on ordinary stable solutions of glucose in water.

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LXXI. URIC ACID AND ALLANTOIN EXCRETION AMONG OFFSPRING OF DALMATIAN HYBRIDS.

By the late HERBERT ONSLOW.

From the Biochemical Laboratory, Cambridge.

Report to the Medical Research Council.

In a previous communication [Onslow, 1923] evidence was given for the fact that hybrids, from a cross between the Dalmatian hound and a terrier, excrete uric acid and allantoin in relatively the same proportion as in the normal dog. The appearance of the two hybrids, a dog and a bitch, was there described, the former being without spots, the latter only very slightly spotted.

In the late autumn of 1922, the hybrid dog and bitch were mated. Six puppies were born on December 26th, 1922. With the purine metabolism of these, the present communication is concerned. Photographs of five of the puppies, *A*, *B*, *C*, *D* and *E*, at the age of about 5 months, are shown in Plate VIII. Two of the puppies were reared only with great difficulty[1], and one, a male, died at the age of 8 weeks. Three of the puppies developed spots, namely, *D*, *E* and the one that died. The spots did not begin to appear until some 3 or 4 weeks after birth.

The bitch *A* (Plate VIII, Fig. 1) resembles a terrier; the ears are yellow and she has a yellow patch round one eye with a slight development of black pigment. The bitch *B* (Plate VIII, Fig. 2) also resembles a terrier; she has brindled ears and patches round the eyes. The bitch *C* (Plate VIII, Fig. 3) is without pigment except for a small black spot or two on the nose and a small yellow patch on the ear. The bitch *D* (Plate VIII, Fig. 4) resembles a true Dalmatian, being spotted more or less regularly over the body and more heavily on the ears. The bitch *E* (Plate VIII, Fig. 5) is a good deal spotted, but not so regularly as *D* and has also black ears, black eye patches, a black patch on the right fore flank and one on the tail.

The puppies were trained for the dog cage early, and the method of procedure was as described in the previous paper, except that the duration of time in the cage did not exceed 12 hours. In the case of all the animals, two preliminary estimations of uric acid were performed before the estimations of both uric acid and allantoin were made simultaneously. The diet was chiefly puppy biscuit and was much less varied than in the case of the parents previously described.

[1] The estimations in the present (as in the previous) paper were carried out by Mr John Lowndes, assistant to the late Mr Onslow. The success of the research is entirely due to Mr Lowndes' skilful and unfailing care for the puppies. (M.W.O.)

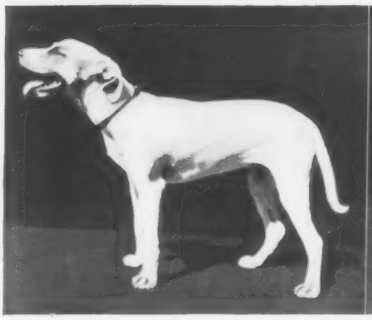


Fig. 1. Bitch *A*.

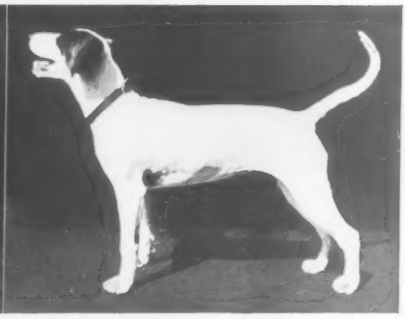


Fig. 2. Bitch *B*.

Fig. 3. Bitch *C*.

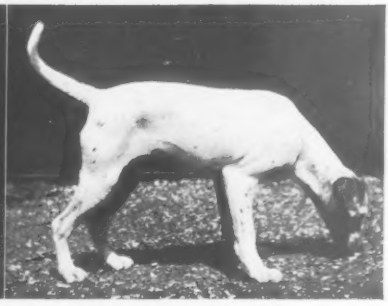


Fig. 4. Bitch *D*.

Fig. 5. Bitch *E*.

The results are recorded in Table I. *Bitch A* was 12 weeks and 6 days old at the date of the first experiment, and 17 weeks and 1 day at the date of the last experiment. At the beginning of the experiments she weighed 3·2 kilos. (7 lbs.), and at the end, 4·5 kilos. (10 lbs.). The duration of time in the cage for Exps. 1 and 2 was 8 hours, and for Exps. 3 and 4, 12 hours.

Table I.

Bitch *A*.

Date of experiment 1923	Vol. of urine cc.	Sp. gr. of urine	In whole volume of urine: Total N g.	Uric acid N g.	Allantoin N g.	Urico-lytic index	Percentage of total N as: Uric acid N	Allantoin N
1. March 26	83 (100)*	—	0·747 74†	0·001 65†	—†	—	0·22	—
2. April 6	53 (100)	—	0·766 00	0·000 94	—	—	0·12	—
3. „ 20	132 (200)	1·020	1·474 54	0·004 24	0·311 32	99	0·29	21·13
4. „ 25	120 (200)	1·025	1·533 14	0·002 52	0·329 10	99	0·16	21·46
			Average for Exps. 3 and 4 only:			99	0·22	21·29

Bitch *B*.

1. March 27	64 (100)	—	0·581 36	0·001 73	—	—	0·30	—
2. April 9	92 (100)	—	0·780 20	0·001 89	—	—	0·24	—
3. „ 16	142 (200)	1·020	1·392 40	0·004 73	0·288 08	98	0·34	20·69
4. May 2	172 (200)	1·021	2·166 06	0·006 92	0·457 23	98	0·32	21·11
			Average for Exps. 3 and 4 only:			98	0·33	20·90

Bitch *C*.

1. March 20	30 (50)	—	0·332 27	0·002 33	—	—	0·70	—
2. „ 28	64 (100)	—	0·729 48	0·002 52	—	—	0·34	—
3. April 11	75 (150)	—	1·158 56	0·005 40	0·220 46	98	0·47	19·03
4. „ 30	206 (250)	1·016	1·418 29	0·006 09	0·294 17	98	0·43	20·74
5. May 11	210 (250)	1·012	1·506 29	0·002 36	0·264 87	99	0·16	17·58
			Average for Exps. 3 and 4 only:			98	0·45	19·88

Bitch *D*.

1. March 21	32 (50)	—	0·464 16	0·002 64	—	—	0·57	—
2. April 4	43 (50)	—	0·617 24	0·002 27	—	—	0·37	—
3. „ 23	170 (200)	1·015	1·204 96	0·003 77	0·258 78	99	0·31	21·48
4. May 9	145 (200)	1·021	1·919 92	0·005 03	0·386 90	99	0·26	20·15
5. „ 15	65 (200)	1·066	0·773 69	0·000 94	0·088 33	99	0·12	11·48
			Average for Exps. 3 and 4 only:			99	0·28	20·81

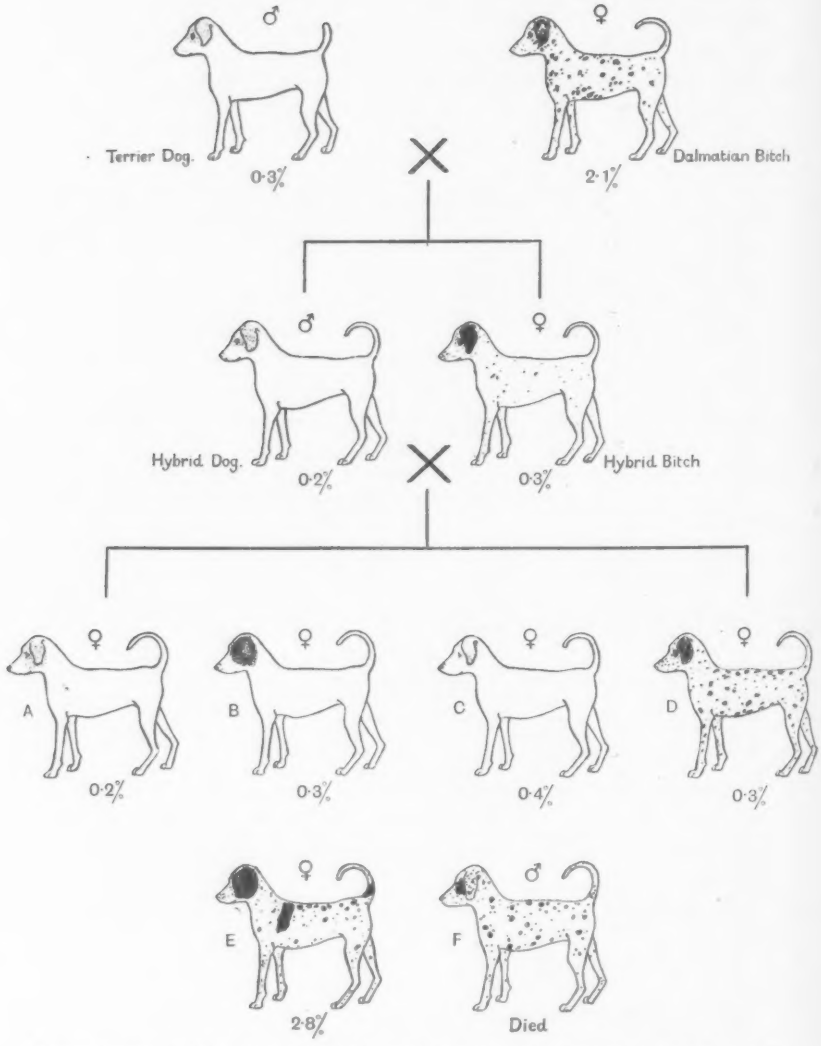
Bitch *E*.

1. March 23	72 (100)	—	0·291 22	0·012 21	—	—	4·19	—
2. April 5	85 (85)	—	0·561 42	0·013 37	—	—	2·38	—
3. „ 18	120 (200)	1·021	1·386 63	0·042 46	0·235 34	85	3·06	16·97
4. May 7	160 (200)	1·020	1·580 02	0·039 31	0·270 50	87	2·49	17·12
5. „ 14	185 (200)	1·020	1·908 21	0·049 38	0·288 08	85	2·59	15·10
6. „ 16	160 (200)	1·025	3·076 28	0·051 89	0·258 78	83	1·69	8·41
			Average for Exps. 3 and 4 only:			86	2·77	17·04

* Figures in brackets denote volume of first dilution.

† Unfortunately four figures only were given in these columns in the previous paper [Onslow, 1923]. The percentages, however, were calculated on six figures and are therefore correct and strictly comparable with those given in the present paper.

Bitch B was 13 weeks old at the date of the first experiment, and 18 weeks and 1 day at the date of the last experiment. At the beginning she weighed 2·7 kilos. (6 lbs.) and at the end 4·5 kilos. (10 lbs.). The duration of time in the cage for Exps. 1 and 2 was 8 hours, and for Exps. 3 and 4, 12 hours.



Scheme showing the inheritance of the Dalmatian characters in the first and second generations. The numbers denote percentages of uric acid nitrogen of total nitrogen.

Bitch C was 12 weeks old at the date of the first experiment, and 19 weeks and 3 days at the date of the last experiment. At the beginning of the experiments she weighed 4·1 kilos. (9 lbs.); at the end 6·6 kilos. (14½ lbs.). The time in the cage for Exps. 1 and 2 was 8 hours, and for Exps. 3, 4 and 5, 12 hours.

During Exp. 5 the animal refused to eat; hence the results are not strictly comparable with the previous experiments and will be considered later.

Bitch D was 12 weeks and 1 day old at the date of the first experiment, and 20 weeks at the date of the last experiment. At the beginning of the experiments she weighed 2·7 kilos. (6 lbs.), and at the end 4·5 kilos. (10 lbs.). The time in the cage for Exp. 1 was 8 hours; for Exp. 2, 7 hours; for Exps. 3 and 4, 12 hours and for Exp. 5, 13½ hours.

Again, Exp. 5 was not comparable to the others, as the animal refused to eat while in the cage.

Bitch E was 12 weeks and 3 days old at the beginning of the experiments, 20 weeks and 1 day at the end. She weighed 3·6 kilos. (8 lbs.) at the beginning of the experiments and 6·3 kilos. (14 lbs.) at the end. The time in the cage for Exps. 1 and 2 was 8 hours and for Exps. 3, 4, 5 and 6, 12 hours. Exp. 6 took place after a diet of water biscuit for 24 hours.

A practice was made of feeding the animals while in the cage. Towards the end of the experimental period, three, namely *C*, *D* and *E* refused to eat. It will be seen, from Exps. *C* 5, *D* 5 and *E* 5 that starvation reduced the percentage of allantoin. Further, for Exps. 1 and 2 in each case the animals were very young. *Hence the average of Exps.* 3 *and* 4 *is considered the most representative for the normal animal in each case.* They are recapitulated as follows:

	Percentage of total N as	
	Uric acid N	Allantoin N
A	0·22	21·29
B	0·33	20·90
C	0·45	19·88
D	0·28	20·81
E	2·77	17·04

In regard to the allantoin excretion, the amounts excreted (except in the starvation experiments) were much more constant than in the case of the parents [Onslow, 1923]. This is probably due to the fact that the diet was more constant, the animals being fed almost entirely on biscuit. Starvation, either self-imposed or designed, as in Exp. *E* 6, reduces the allantoin percentage. At the same time it also reduces the uric acid percentage but does not appear to reach the condition, described by Benedict [1916], in which the animal may excrete a higher percentage of uric acid than of allantoin.

From the urine of the bitch *E*, uric acid crystals separated on adding 10 % hydrochloric acid and allowing to stand.

In regard to the uric acid excretion, it is clear, from the results in the second generation, that there has been a segregation of the character involving the power to destroy this substance from the character involving lack of this

power. Apart from this outstanding fact, it is difficult to postulate the exact course of inheritance from the data given by this one family alone. The results, however, suggest that, in main outline, the grandfather terrier (probably of mixed breed) carried a dominant factor for destruction of uric acid; also a factor for the inhibition of spotting which is not always entirely dominant in the hybrid. If it is assumed that these two factors segregate independently, then it is a simple two-factor Mendelian case (though there are, without doubt, subsidiary complications). Sixteen individuals would then, theoretically, be of the following kinds:

Four unspotted dogs; of these one only (*i.e.* 1 in a total of 16) should produce uric acid.

Twelve more or less spotted dogs; of these four are pure bred and eight are hybrid. Of the four pure bred, one only (*i.e.* 1 in the total 16) should produce uric acid. Of the hybrid spotted, two (*i.e.* 2 in the total 16) should produce uric acid.

Only a certain number, of course, of the above animals of different composition would appear in a small litter and the proportions, moreover, would not necessarily be in close agreement with expectation.

It is suggested that the bitches *A*, *B* and *C* belong to the unspotted class; the bitch *D*, which closely resembles a pure Dalmatian, to the pure spotted class which does not produce uric acid; and, finally, the bitch *E* to the hybrid spotted class which does produce uric acid.

The accompanying scheme (p. 566) sets forth the inheritance of the two characters in the first and second generations. The criticism may be made that the animals in the scheme are represented as more heavily spotted than is justified by the photographs. Observation, however, demonstrates the fact that the spots, though slight in the young animal, increase in size and intensity, month by month, until the animal is 18 months or even two years old. The scheme is therefore designed to show, as correctly as possible, the markings of the mature animals.

In regard to the question as to whether the purine metabolism might not alter further with age, it may be pointed out that, at the completion of their experiments, the dogs of the second generation were approximately the same age as the dogs of the first generation at the beginning of their experiments. The metabolism of the latter did not change during the subsequent three months of experimental work.

Further matings and experiments no doubt would yield interesting results, but the main object of the research as formulated by the late author, namely, the mode of inheritance of the uric acid-producing character through two generations, has been determined.

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LXXII. THE EFFECTS OF VITAMIN-DEFICIENT DIETS ON THE ADRENALINE EQUILIBRIUM IN THE BODY.

By LOUIS GROSS

Beit Memorial Research Fellow.

From the Laboratories of the Royal College of Surgeons, London, and the Institute of Physiology, University College, London.

(*Received July 4th, 1923.*)

(A) Introduction.

In 1919 McCarrison [1919] found that certain vitamin-deficient and otherwise unbalanced diets produced a marked increase in the size of the adrenal gland, which increase was associated with a larger store of adrenaline in the case of vitamin *B* deficiency. In fact he was at first inclined to believe that the appearance of oedema in the wet form of beriberi was to a large extent due to increased intracapillary pressure following on an increased output of adrenaline. In 1914 Funk and v. Schönborn [1914] found an increase in the blood sugar of pigeons fed on polished rice and in 1920 Funk [1920] suggested that it was due to increased adrenaline output. Anrep[1] observed in one or two cats which had been fed for a considerable time on vitamin *B*-deficient food that the blood-pressure was approximately 285 mm. Hg. while the animals were under anaesthesia. I have shown elsewhere[2] that deprivation of vitamin *B* from the diet of the adult rat leads almost at once to a pronounced intestinal stasis. These observations as well as the fact that adrenaline is a powerful gut inhibitor and that rats under advanced vitamin *B* deficiency show a number of signs resembling to some extent the effect of an increase in a sympathomimetic substance (*e.g.* nervousness, excitability, hair standing on end, etc.) made it seem very possible to me that the whole complex could be explained on a basis of hyperadrenalinemia [Gross, 1922]. Upon such a supposition, too, an explanation was afforded for the accumulation of large pigment-bearing cells confined to the subepithelial stroma of the colon which Keith [1914] described as occurring in advanced stasis in the human. For, granted that this pigment was taken in by phagocytes, or in some way obtained from the colon, (probably as a result of breaking down of aromatic decomposition

[1] Private communication.

[2] This will appear in a forthcoming publication.

products as suggested by Pick) these swollen pigment-bearing cells would find it impossible to pass lymphatics which were partially choked off by a muscularis mucosae kept in tonic contraction by increased adrenaline [Gunn and Underhill, 1914]. This explained, too, the lymphatic lakes and "soggy colon" of chronic intestinal stasis.

The experiments about to be described represent an attempt to confirm some of the statements made by these observers, and to determine directly the adrenaline content of the blood. 319 rats were used for the purpose.

(B) The Relation of Body Weight to Adrenal Gland Weight and Adrenaline Store.

The greater number of observations published on this subject are based on adrenal glands taken from animals which were either dead or dying. Furthermore, the diets largely used were grossly unbalanced so that the results in any case cannot be held to be those following pure vitamin deficiencies. The question seemed therefore worth reinvestigating in order to determine the changes in these glands which follow pure vitamin deficiencies on otherwise well balanced diets, and to make the adrenaline determinations on glands from adult rats which show evidences of vitamin deprivation, but are, on the whole, still in a relatively fair condition of health.

Table I.

Table of the diets used, showing the proportions of the ingredients in parts by weight. The butter was mixed with 2 % of its bulk of cod-liver oil. The heated casein was prepared by heating shallow layers of casein in a dry oven for 24 hours at a temperature of 110° C. The extracted casein was prepared by extracting casein with boiling 95 % alcohol for 4 hours. "Marmite" is a commercial preparation of autolysed yeast. The hardened fat used was hydrogenated cotton seed oil. The salt mixture was as follows in parts by weight:

Sodium chloride	5·19	Calcium lactate	39·00
Magnesium sulphate	7·98	Ferric citrate	3·54
Sodium acid phosphate	10·41	Calcium phosphate	16·20
Potassium phosphate	28·62		

Diet	Casein 20 parts	Starch 50 parts	Hardened fat, or butter, 10 parts	Salt mixture 5 parts	Marmite 5 parts	Lemon juice 5 parts	Water 30 parts
P	Casein	,,	Butter	,,	,,	,,	,,
A	Heated	,,	Hardened fat	,,	,,	,,	,,
B	Extracted	,,	Butter	,,	None	,,	,,
C	Casein	,,	Butter	,,	Marmite	None	,,

The diets used in these experiments are shown in Table I. The average time during which the rats were kept on the various diets, as well as their average weights at the beginning and end of the experiment, are shown in Table II.

The rats were killed by a sharp blow on the head with a metal rod immediately followed by amputation of a fore limb. The blood was caught in a clean glass receptacle to be subsequently used for various experiments. This method of killing, besides being even more humane than coal-gas poisoning, gives an excellent yield of clean uncontaminated blood. The tissues are of course unaltered for histological purposes.

Table II.

The rats on *P* diet showed a steady increase in weight. The rats on *B* diet showed a consistent fall in weight. The rats on *A* and *C* diets presented a flattened growth curve which was more exaggerated in the case of *A* diet. The figures which give the average weights at the end of the experiment in this table include an average final fall in weight of 10 g. in the case of *A* diet, and an average final fall of 8 g. in the case of *C* diet.

	P diet	*A* diet	*B* diet	*C* diet
Average time on diet, in weeks	8	6	5	7
Average weight at beginning of experiment	184	205	228	193
Average weight at end of experiment	219	207	194	204

The method employed for estimating the adrenaline content of the adrenal glands was that of Folin, Cannon and Dennis [1912], which was largely used and found quite satisfactory by McCarrison [1921] and by Kellaway [1920]. The only modification which I adopted was to add the uric acid solution to my adrenaline extract in a quantity equal to that used in the standard tube. In this way the estimation of the small quantities of adrenaline found in a rat's adrenal glands was made possible. I found that the end-point in the colorimetric readings could be made considerably sharper by interposing an orange-coloured screen between the mirror of the colorimeter and the source of light. The latter should preferably be artificial. I used an Osram Daylight bulb, employing this light reflected from milk glass. The adrenal glands were obtained immediately after death and carefully dissected free from the surrounding fat. They were then weighed and the adrenaline estimated.

The results in this investigation are the averages from 350 glands obtained from 175 rats. A number of mice were also employed, but the experimental error was found too great to render these observations of any importance. Table III has been constructed entirely on the figures from rats' glands.

Table III.

	P	*A*	*B*	*C*	*B* deficiency followed by *P* diet
(*a*)	0·0253	0·0221	0·0188	0·0194	0·0174
(*b*)	0·0253	0·0228	0·0217	0·0208	0·0174
(*c*)	1·75	1·82	2·03	1·71	3·88
(*d*)	0·0442	0·0403	0·0382	0·0332	0·0675
(*e*)	0·0442	0·0415	0·0440	0·0356	0·0675

(*a*) represents g. of adrenal gland per 100 g. of highest body weight of rat.
(*b*) represents g. of adrenal gland per 100 g. of final body weight of rat.
(*c*) represents milligrams of adrenaline per g. of adrenal gland.
(*d*) represents milligrams of adrenaline per 100 g. of highest body weight of rat.
(*e*) represents milligrams of adrenaline per 100 g. of final body weight of rat.

Row (*a*) in Table III shows the comparison in weight between the glands of normal and vitamin-deficient rats. The numbers are all reduced to grams of gland per 100 g. of highest body weight of rat. The first fact to be noticed is that the adrenal glands of rats on the different vitamin-deficient diets decrease in size. This is most marked in vitamin *B* deficiency and least in vitamin *A* deficiency. Rondoni [1914, 1915], McCarrison [1919] and others [see McCarrison, 1921] have shown that vitamin and other dietary deficiencies cause a considerable increase in the size of the adrenal glands. McCarrison

conducted his experiments on monkeys, guinea-pigs and pigeons. My figures indicate the complete opposite for adult rats on pure vitamin deficiencies. The difference must depend either on the different species used, or the unbalanced diets used by these observers, or on changes during the last stages of vitamin deficiencies. Very likely the second reason given is the most important. I have noticed that the adrenal glands appeared noticeably enlarged and congested in several rats which I had deliberately kept on vitamin *B* deficiency to the point of death. The last stages of vitamin *B* deficiency are, however, undoubtedly complicated by the results of inanition. The average duration of my rats on vitamin *B*-deficient diet from which these figures are taken, is 5 weeks. One other point is worth noticing. In the normal rat, the adrenal gland weight is not in linear proportion to body weight. The heavier rats have as a rule slightly smaller adrenals per 100 g. of body weight than have the lighter or smaller rats. Attention must be paid to this point in comparing the effects of the various experimental diets. Table II shows that there is comparatively little difference between the average weights of my animals.

Row (*b*) represents grams of adrenal weight computed per 100 g. of final weight of rat. These figures show that the atrophy of the adrenal gland is not only absolute but relative as well.

Row (*c*) represents milligrams of adrenaline per g. of adrenal gland. It is seen that the adrenaline content of the adrenal glands of vitamin *B*-deficient rats shows a moderate relative increase in the store of adrenaline per weight of gland as compared to the content of adrenal glands from normal, *A*- and *C*-deficient animals. When, however, the gland content of adrenaline is reckoned per 100 g. of highest body weight of rat (row (*d*)), it is seen that an absolute diminution has taken place in vitamin *B*-deficient rats and even more so in *C*-deficient rats. Finally, when the adrenaline in the glands is computed against final body weight, which is nearest to the relation which it had during the life of the rat while on the experimental diet, it is seen that even these differences largely disappear (row (*e*)). *C* diet is the only deficiency which appears to produce any appreciable change. This change, however, is of the order of 19 %, and in view of the relatively high technical errors involved in determinations of this kind, too much significance must not be attached to it.

An entirely unexpected result was the relatively small glands found in rats which had been on a vitamin *B*-deficient diet for an average of 5 weeks and then placed on normal diet until the normal weight had been regained (usually about $2\frac{1}{2}$ weeks). The average weight of gland per 100 g. of rat was 0·0174 g. The adrenal content was extremely high, 3·88 mg. per g. of gland. Worked out in term of mg. of adrenaline per 100 g. of highest weight of rat which was in this case equal to final weight, we arrive at the astonishing figure of 0·0675. This represents an absolute increase of 53 %.

Other points brought out were: (*a*) Unilateral adrenalectomised rats showed an increase in the store of adrenaline in the remaining gland. Thus,

whereas the normal gland showed 1·75 mg. the gland remaining after unilateral adrenalectomy contained 2·34 mg. of adrenaline per g. of gland. (*b*) Daily injections of adrenaline seemed to discharge, or perhaps decrease the formation of adrenaline in, the glands to a moderate extent, causing them to contain approximately 13 % less than on the same diet without the adrenaline injections. This was true for both normal and vitamin *B*-deficient diets.

Microscopic examination of the adrenal glands from normal rats as well as from those deficient in vitamins *A*, *B* and *C* respectively showed no appreciable change. The fixatives and stains used were 10 % neutralised formalin in saline, and osmic vapour [Cramer, 1918]. This investigation therefore shows that if the diet is otherwise well balanced, and if the adult rat is not kept on this diet until moribund, the changes in the adrenal glands both anatomically and functionally are comparatively slight.

(C) Attempts to determine the amount of Adrenaline present in the Blood and Serum of normal and Vitamin-deficient Rats.

This investigation was carried on at the same time, and on the same animals, as were used for the experiments described in the preceding section. It was not until the completion of the latter experiments that the statistical evidence derived therefrom pointed to the fact that when the store of adrenaline is compared to the weight of the animal, it is found that pure vitamin deficiencies produce on the whole very little, if any, disturbance in the adult rat. When the studies about to be described were being carried out, therefore, it was still on the assumption that vitamin deficiencies cause an upset in the adrenaline equilibrium with probably a resultant hyperadrenalinemia in the case of vitamin *B*.

The methods which I employed were as follows:

(*a*) Effect of serum on the isolated frog's pupil.

(*b*) Effect of blood and its derivatives on various other isolated organs such as rat's and rabbit's uterus and intestine, perfused rabbit's ear and perfused frog. In this study I co-operated with Professor A. J. Clark [unpublished].

It is, unfortunately, impossible to isolate adrenaline from the blood at present. Attempts at ultrafiltration and subsequent precipitation resulted in failure, I therefore turned my attention first to the use of the isolated frog's pupil. This method, which was first suggested by Meltzer and Auer [1904] and of which a critical survey was made by Schultz [1909], is not a specific test for the presence of adrenaline, for the mydriasis caused by this substance can also be brought about by other substances probably normally present in blood, *e.g.* pituitary extract. Nevertheless, in the hope that some idea of the relative amounts of mydriatic substance present in the serum would furnish useful evidence on the question, 63 rats were used for this purpose.

The blood was obtained in the manner already described. It was received

into a centrifuge tube and immediately spun for 5 minutes at 4000 revolutions per minute. The separated serum was pipetted off and 0·35 cc. run into each of several dwarf test-tubes. Frogs had meanwhile been pithed, the eyeballs carefully removed and placed upon a sheet of white paper with the pupils facing a constant source of light. It was planned to expose these pupils to the light for 5 minutes. The two diameters of the frog's elipsoidal pupil were spanned with a pair of tool-maker's dividers and measured on a Vernier scale. The eyeballs were then immersed, one in each tube of serum. The same measurements were taken a half-hour later and one hour later, care being taken to carry out the whole process under similar conditions.

The areas of the pupils were computed from the measurements obtained, and the mydriatic effect of the serum expressed in terms of per cent. of the area increase over that found in the pupil just before immersion into serum. For controls, sera from normal rats were used.

A separate series of control experiments had brought out the following points:

(*a*) There was a well-marked variation in the reaction of the different frogs' pupils to the same serum, sometimes even the two eyes of the same frog gave a different reading for the same serum.

(*b*) On the whole the average of a fair number of eyes gave figures for known concentrations of adrenaline added to saline, which showed a distinct diminution in mydriatic power with increased dilution of the adrenaline. The utmost sensitivity, which I could obtain by this method, was a reaction to 1 in 8,000,000 adrenaline hydrochloride in saline.

Table IV.

Per cent. of area increase in frog's pupil: (*a*) after one half hour immersion in serum; (*b*) after one hour immersion.

	P	*A*	*B*	*C*	*B* deficiency followed by *P* diet
(*a*)	39	23	75	42	114
(*b*)	25	29	49	22	90

Table IV shows the results of these tests. It is seen that the serum of vitamin *A*-deficient rats has a somewhat lower mydriatic power, and that of vitamin *B*-deficient rats a considerably higher power, as compared to the controls. Also *B* rats which were subsequently fed on *P* diet for about two weeks showed an even greater mydriatic power of the serum. It will be remembered that the adrenaline content of these glands was considerably increased. This, in fact, is the only relation which I could find between the mydriatic power of the serum and the adrenaline store in the glands. Other points not shown in the table were the considerable decrease in mydriatic power of serum from unilateral adrenalectomised rats. The serum from rats which had daily injections of adrenaline showed neither in control nor in vitamin *B*-deficient rats, a departure from that found in the normal. In view

of the individual variations, the latter experiments as well as those on vitamin *A*-deficient animals, were rather too few in number to warrant more than a possible suggestion for future investigation.

It is seen, therefore, that if this increase in mydriatic power in the serum from vitamin *B*-deficient rats is due to adrenaline, the concentration in the serum should be well over 1 in 8,000,000.

To test this, Professor Clark and I compared the effects of fresh blood within 20 seconds from being shed, defibrinated blood, serum, plasma, ultrafiltrate of serum and plasma, and alcoholic and ether extracts of serum and plasma on the isolated organs previously mentioned in this section. Considering the fact that some of the organs, for example, rat's uterus, were occasionally sensitive to 1 in 500,000,000 adrenaline, well-marked reactions should have been obtained. It is true that shed blood very quickly develops vaso-constrictor and smooth muscle stimulating substances, but we endeavoured to obtain at least a quantitative difference between the blood of normal and vitamin *B*-deficient animals which would indicate a higher content of adrenaline in the blood of one of these groups.

Our results, which are described in greater detail in a separate publication, merely demonstrated the enormous variability of these fluids and tissues. We were, in short, unable to establish any definite differences between the adrenaline content of the blood of the normal and vitamin-deficient animals. Nor were we able to find any other characteristic differences in the properties of the blood of these animals in their effects on the isolated organs tested. It seems, therefore, highly improbable that the increased mydriatic effect of the serum which I found in vitamin *B*-deficient rats was due to a higher concentration of adrenaline.

(D) Investigations on the Carbohydrate Metabolism of Vitamin *B*-Deficient Rats.

The chief interest which I had in the question was in so far as it would furnish indirect evidence on the relative amount of adrenaline in the circulation. Mr P. Eggleton and I are, however, making a detailed study of the carbohydrate metabolism in *B*-deficient rats which we hope will be published shortly. The present preliminary notice is based on the results from 48 rats. On the whole these are negative.

In 1914 Funk [1914] suggested that the anti-beriberi vitamin plays some part in carbohydrate metabolism. He based this view upon the fact that addition of carbohydrate to a standard beriberi diet caused the symptoms to appear earlier in pigeons. In the same year Funk and v. Schönborn [1914] found supporting evidence for this hypothesis in the discovery of a hyperglycemia and low liver glycogen in beriberi pigeons. They also observed that the administration of yeast tended to lower the blood sugar and increase the store of glycogen in the liver. In criticising these results, Vedder [1918] pointed out that overfeeding was probably the determining factor. In 1919

McCarrison [1919] stated that the absence of "anti-neuritic" food factors from the diet leads to an increase in the weight and adrenaline load of the adrenal gland and to a state of acidosis due to the imperfect metabolism of carbohydrates and the acid fermentation of starches. How far the increased weight of the adrenal gland and increased adrenaline store is due to deprivation of vitamin *B*, and how much to inanition, has been discussed in section (B). On the basis of McCarrison's results, Funk [1920] pointed out that increased adrenaline in the circulation was probably responsible for the hyperglycemia which he had obtained in another series of beriberi pigeons (bled out under A.C.E. mixture). It is noteworthy that Funk's figures show in this experiment a higher glycogen content in the beriberi livers than is seen in his normal controls. This is, of course, the opposite to what he and v. Schönborn had previously found. Very recently Mattill [1923] studied the respiratory quotient of vitamin *B*-deficient rats and concluded that there was no interference with the process of glucose combustion.

This condensed review presents the chief points brought forward which bear on the relation between vitamin *B* and carbohydrate metabolism. It was obviously important to reinvestigate this question in order to determine:

(*a*) Is pure vitamin *B* deficiency in the rat associated with an alteration in the carbohydrate metabolism?

(*b*) If so, what part in the cycle is affected, and in what manner?

The method which we employed was as follows: a large number of rats were fed on vitamin *B*-deficient diet and at different stages of the deficiency some were used for blood sugar and liver glycogen determinations. Experiments were also made on the blood sugar following the digestion of starch and that following the absorption of glucose. Since it is very difficult to obtain more than one sample of blood sugar from a rat without having recourse to anaesthesia, we killed the rat in each instance and relied upon statistical evidence of numbers for our conclusions. This eliminated most of the artefact which would otherwise be introduced into the actual sugar values. Also, much of the individual variation was thereby brought to a more reliable average. For blood sugar determinations we employed the MacLean [1919] 0·2 cc. method which we found quite reliable. For glycogen determinations we used Pflüger's method [1910], finally determining the hydrolysed sugar by Bertrand's method [1920].

The points investigated were as follows:

(*a*) *Blood sugar and liver glycogen from normal and vitamin B-deficient rats as they were taken out of the cage.* This is of course open to the objection that the blood sugar will depend upon when the animal had its last meal. These determinations were made, however, in order to compare the results with some work published by other authors who do not mention the precaution of preliminary starvation. The results obtained showed no appreciable difference for blood sugar, but a considerably lower liver glycogen in the *B*-deficient rats.

(*b*) *Blood sugar and liver glycogen from rats starved for* 24 *hours.* This is much more reliable since it does away practically entirely with carbohydrate absorption from the bowel. The liver glycogen in both normal and vitamin *B* rats was nil. The blood sugar was slightly higher in the *B*-rats than in the normal (0·09 g. % for normal, 0·117 g. % for *B*). It is possible, however, that additional numbers will reduce or remove this difference.

(*c*) *Rate of absorption of glucose from the alimentary tract of rats starved for* 24 *hours.* A glucose solution of known strength was introduced by catheter into the stomach, and blood sugar estimations were made at 15-minute intervals for one hour. No significant differences were found in the blood sugar curves from both groups of animals.

(*d*) *Introduction of a starch paste* gave us an indication of the rate of digestion as well as of absorption. Again no appreciable differences were found between normal and vitamin *B*-deficient rats.

In none of these experiments was a glycosuria found.

It is seen, therefore, that there is not as yet sufficient evidence to attribute to vitamin *B* deficiency an upset of carbohydrate metabolism, at any rate of the part studied by us thus far. Certainly the changes which we have found are insufficient to account for the marked general disturbances of the animal and do not afford adequate confirmatory support to the theory of hyperadrenalinemia. It is possible, however, that larger numbers of rats may reveal evidence which has not thus far been brought out.

(E) Conclusions.

The general conclusion which can be drawn from these investigations is that pure vitamin deficiencies set up very little alteration in the adrenaline equilibrium (*i.e.* relations between adrenaline store, gland weight and body weight) in the adult rat, providing the rat is not brought to the point of death on the diet. Such alterations as do occur can be explained on a basis of general tissue atrophy. The striking changes which have been recorded by other observers have probably been produced by the accompanying starvation and unbalanced dietary rather than by the deficiency in vitamins. Unless indeed, species so widely different as pigeons, guinea-pigs and monkeys are predisposed to alterations in adrenaline equilibrium to a much greater degree than is the adult rat. This seems very unlikely. Contrary to an upset in adrenaline equilibrium, the adrenaline store in the adrenal gland maintains an extraordinarily constant proportion to the weight of the rat. The suggestion forces itself upon one that this is no mere coincidence and that the remarkable way in which the store of adrenaline follows the body weight in its vicissitudes caused by the vitamin deficiencies argues very strongly for a finely controlled mechanism. It is then perhaps not surprising that I have been unable to obtain either direct or indirect supporting evidence of an unmistakable alteration in the adrenaline content of the blood.

I wish to tender my sincerest thanks to Sir Arthur Keith, to Professors E. H. Starling and J. C. Drummond, and to Dr Katherine H. Coward for their generous help and advice, as well as for the privilege of carrying out this work in their laboratories.

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LXXIII. THE OXYGEN CONTENT OF METHAEMOGLOBIN.

By HERBERT ELDON ROAF
and WILLIAM ARTHUR MERRETT SMART.
Eliza Ann Alston Scholar, London Hospital Medical College.
From the Department of Physiology, London Hospital Medical College.

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Some years ago one of us made measurements of the osmotic pressure of haemoglobin with a view to correlating changes in osmotic pressure of haemoglobin with haemolysis [Roaf, 1910, 1, and 1912]. In certain cases, notably in the action of alkali, there was a distinct relation between the effect of alkali on haemolysis and on the osmotic pressure of haemoglobin. In the case of acid, however, the results did not show so great an increase in osmotic pressure and it was suggested that some secondary change might have occurred.

The results were explained on the basis that haemoglobin acted as an amphoteric electrolyte with the formation of ionising salts, thus producing a corresponding rise of osmotic pressure and the establishment of an electrical potential.

Recently Mrs Wilson [1923] has published the results of experiments with carbon dioxide and acetic acid which confirm the result that acid causes a rise of osmotic pressure and she obtained higher pressures per 1 % of haemoglobin than were obtained by Roaf. Unfortunately, her paper does not indicate that spectroscopic examination, accompanied by reduction, was made when the pressure was high. In view of the fact that haematin may be recombined with protein [Menzies, 1894, 2], it is possible that the highest pressures obtained were due to some splitting of the haemoglobin and the subsequent fall due to recombination. It was for fear of this condition that dialysis experiments were not carried out with haemoglobin, but Mrs Wilson overlooks the fact that such experiments were carried out with serum proteins [Roaf, 1910, 2][1].

[1] Mrs Wilson whilst confirming my results and agreeing with my conclusions states that they were based on slight evidence. The evidence was that haemoglobin although showing no evidence of decomposition into haematin gave higher osmotic pressures than could be accounted for by single molecules of haemoglobin. This evidence is good enough for the guarded statement: "It is most likely that the correct explanation is that haemoglobin forms ionising salts with alkalies and acids, and that the observed pressures are due to the sum of the pressure caused by haemoglobin and the ions into which the salt can split, for instance sodium and 'haemoglobyl' ions."

The view that proteins form ionising salts is based on a much wider basis than one set of experiments. The starting point for this belief is the work of Emil Fischer in showing that

In the previous experiments methaemoglobin was tentatively considered to be the stable form of oxyhaemoglobin on the acid side of the isoelectric point although that view did not harmonise with the formation of alkaline methaemoglobin.

Buckmaster [1914] has published results in which he found that methaemoglobin formed by the action of hydroxylamine yields with hydrazine hydrate only half the nitrogen that is furnished by oxyhaemoglobin. The bearing of this point on the osmotic pressure measurements is that acid oxyhaemoglobin may have a high osmotic pressure but it readily passes into methaemoglobin with a lower osmotic pressure. If one could measure the osmotic pressure of oxyhaemoglobin in acid before methaemoglobin was formed, sufficient rise of pressure might be recorded to account for haemolysis by acid.

We thought this subject of sufficient interest to merit investigation by a different method. We measured the amount of oxygen given off when methaemoglobin was formed by the action of acetic acid on oxyhaemoglobin and compared this with the amount given off when another portion of the same oxyhaemoglobin solution was acted on by potassium ferricyanide.

Experimental Methods.

The method finally adopted was to use the principle of van Slyke's blood gas apparatus. In order to carry out a series of observations we used Haldane sampling bulbs for the containers. A tube over 76 cm. long was used to produce the vacuum (Fig. 1). On the upper end of this tube was a bulb (*B*) to receive the solution on evacuation. The lower end of the tube (*A*) was furnished with pressure tubing and mercury bulb in the usual manner. The Haldane sampling tube (*C*) was united with the bulb (*B*) by pressure tubing. After testing the apparatus to see that the taps did not leak the whole was filled with mercury. The haemoglobin solution was run in from a pipette through the upper opening of the sampling tube. The pipette was filled above the mark and the excess allowed to escape through the side outlet of the Haldane tube, so that all the connections up to the tap were filled with the solution. The tap was then turned through 180° so that the haemoglobin solution ran into the sampling tube above the mercury.

The connections were washed out with water through the side tube and the reagent added in the same way that the haemoglobin solution was added.

proteins are composed of amino acids. Other evidence was briefly quoted in the paper to which Mrs Wilson refers. She evidently overlooks the large amount of work published in this Journal from the Laboratory of the late Professor Benjamin Moore, F.R.S., which shows that the inorganic constituents are an integral part of the protein when they increase the osmotic pressure of the protein, otherwise the inorganic constituents would escape through the membrane. This work has been corroborated by other observers.

One point in connection with that work is frequently overlooked, viz. the importance of changes in the osmotic pressure of colloids on cell mechanics. If other cell proteins are as easily affected as haemoglobin considerable effects may be produced in cells by changes in osmotic pressure.

When ferricyanide was used the apparatus was immediately evacuated. After evacuation was completed the solution was collected in the bulb (*B*), the lower tap of the sampling tube was turned and the solution run out through the lower side tube for spectroscopic examination. The lower tap was then reversed and the mercury run into the Haldane tube.

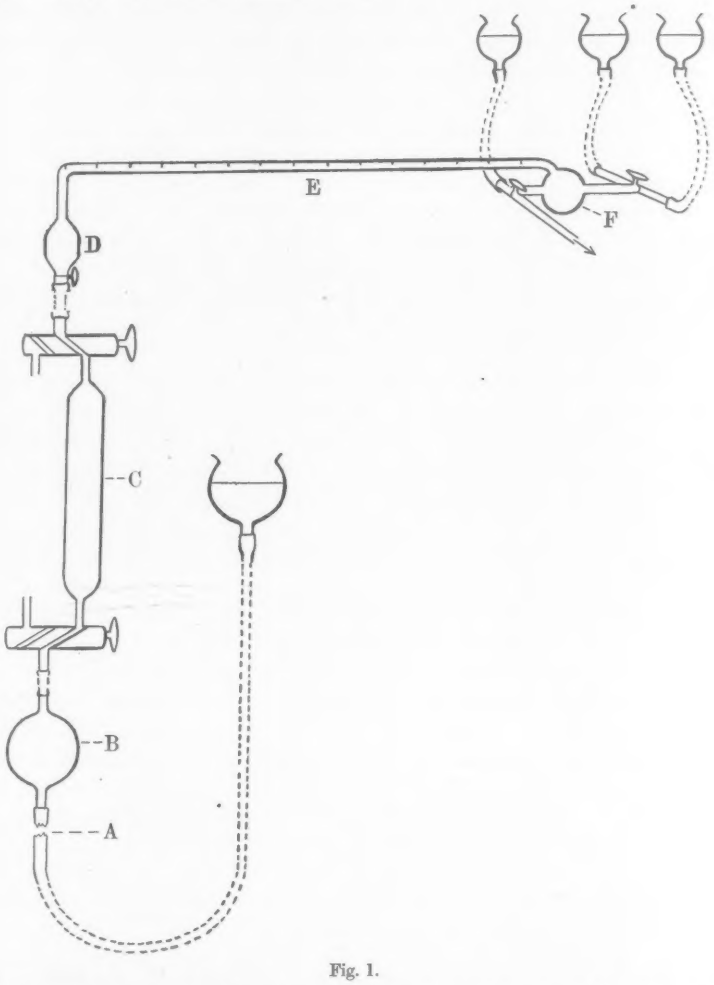

Fig. 1.

When acid was used, the reagent was added to the haemoglobin, both taps were closed, the tube disconnected, and left (usually for twenty-four hours at room temperature) until the acid had converted the oxyhaemoglobin into methaemoglobin. It was then re-connected and evacuated as described above.

The gas was analysed by running it into a tube containing alkaline water. This tube was connected to the Haldane tube by pressure tubing and the connections filled with water by running the alkaline solution through the upper side outlet of the Haldane tube. The upper tap was then reversed and by raising the mercury the gas was forced into the bulb (*D*).

By disconnecting the analysing tube from the Haldane tube and attaching a rubber tube with reservoir to the end which had been connected to the Haldane tube the gas can be forced along the measuring tube (*E*).

The absorption of gas was carried out in the far bulb (*F*). This bulb is furnished with two side tubes each with a right-angled tap so that either of two supply tubes, making four in all, could be connected with the bulb. The first reservoir contained the alkaline water for manipulation of the gas along the measuring tube. The second contained saturated sodium hydroxide for absorption of any carbon dioxide which escaped absorption by the alkaline water. The third contained concentrated pyrogallol which when run into the strong alkali in the bulb absorbed the oxygen. The fourth opening was attached to a waste tube for the purpose of washing out the apparatus.

The amount of oxygen given off was measured by the difference in volume after the action of strong alkali followed by alkaline pyrogallol, the usual assumption made in gas absorption analysis.

The haemoglobin solution was prepared as follows. The blood was centrifugalised and the corpuscles twice washed with saline. The corpuscles were haemolysed by an equal volume of distilled water. At first as the result of osmotic pressure experiments we did not dare to use a preservative but later we found that toluene did not interfere with the results but actually improved them.

Caprylic alcohol was added to the alkaline water in the measuring tube to decrease the difficulty due to frothing.

Results.

The results show unmistakably that addition of acid sufficient to convert oxyhaemoglobin into methaemoglobin liberates approximately half the amount of oxygen that would be given off by the same quantity of haemoglobin when acted on by ferricyanide. A few of the results are given in tabular form.

Discussion.

Before deducing that methaemoglobin contains only half the replaceable oxygen that oxyhaemoglobin contains we must examine all other possible explanations. As oxygen is given off the product formed by acid must contain less oxygen than oxyhaemoglobin and there is no source of oxygen other than the oxyhaemoglobin.

Tabular Statement of Results in those experiments in which toluene was used as a preservative.

Date	Amount of haemoglobin solution cc.	Treatment of solutions	Amount of oxygen given off cc.		Percentage of oxygen capacity	Spectrum examination
Exp. 1	10	5 cc. saturated ferricyanide	1·68	av. of 2 readings	100	Methaemoglobin
19. ii. 23	20	1·6 cc. 3*N* acetic acid	1·54	„ 2 „	45·8	Methaemoglobin + a doubtful trace of oxyhaemoglobin
	20	1·6 cc. 3*N* acetic acid after equilibration with air	0·22		6·5	Methaemoglobin + a trace of acid haematin
Exp. 2	10	5 cc. saturated ferricyanide	1·30	av. of 2 readings	100	Methaemoglobin
26. ii. 23	20	1·6 cc. 3*N* acetic acid	1·275	„ 2 „	49	„
	20	1·6 cc. 3*N* acetic acid after equilibration with 90·1 % oxygen	0·57	accounted for by solubility of oxygen	22	„
Exp. 3	10	5 cc. saturated ferricyanide	1·37	av. of 5 readings	100	Methaemoglobin
5. iii. 23	20	1·6 cc. 3*N* acetic acid	1·355	„ 2 „	49·5	„
	20	1·6 cc. 3*N* acetic acid after equilibration with 56 % oxygen	0·42	accounted for by solubility of oxygen	15·3	„
Exp. 4	10	5 cc. saturated ferricyanide	1·53	av. of 3 readings	100	Methaemoglobin
12. iii. 23	20	1·6 cc. 3*N* acetic acid	0·44		14·4	Methaemoglobin + acid haematin
A further set was started next day containing less acid with the following result:						
	20	1·4 cc. 3*N* acetic acid	1·39	av. of 2 readings	45·4	Methaemoglobin
Exp. 5	10	5 cc. saturated ferricyanide	1·15	av. of 2 readings	100	Methaemoglobin
28. v. 23	20	1·4 cc. 3*N* acetic acid	1·22		53	„

The average of these five experiments is 48·5 % of the oxygen capacity is given off when oxyhaemoglobin is converted into methaemoglobin.

We must first consider whether methaemoglobin is formed by the action of acid. There may be several forms of methaemoglobin, but at present it is recognised by its colour and absorption band. We have always examined the solution from the experiments in the following manner. We examined it spectroscopically for a band in the red near the *C* line. The solution was rendered alkaline and the change to alkaline methaemoglobin observed. The solution was then reduced and examined for reduced haemoglobin and reduced haematin. Any experiment the solution from which showed oxyhaemoglobin on first examination or reduced haematin on reduction was discarded.

We observed that the spectrum of methaemoglobin formed by the addition of acid lacked the two lines between *D* and *E* which are usually seen with methaemoglobin formed by ferricyanide. We think, however, that this does not indicate any real difference [compare Menzies, 1894, 1] because the addition of alkali to methaemoglobin formed by acid causes the two bands to appear. In our opinion the methaemoglobin formed by ferricyanide is a mixture of methaemoglobin and alkaline methaemoglobin: the two bands disappear with much less acid than is necessary to form methaemoglobin from oxyhaemoglobin.

The next point is whether the oxygen given off might have come from the incomplete formation of methaemoglobin. Any oxyhaemoglobin left unchanged might give off oxygen on evacuation. This alternative is shown not to occur because in those experiments in which oxyhaemoglobin was recognised spectroscopically the volume of oxygen given off was less than when no oxyhaemoglobin was found.

The alternative difficulty, namely, that acid haematin may be formed with the liberation of oxygen is negatived by the result that if reduced haematin is seen after reduction the yield of oxygen is lower (see Exp. 4). In fact we made a few experiments to see if acid haematin contained less oxygen than oxyhaemoglobin. We found that if acid haematin is formed by using larger quantities of acid or by acid plus pepsin no oxygen is given off, thus indicating that acid haematin contains as much oxygen as oxyhaemoglobin.

In some experiments in which we treated oxyhaemoglobin with acid over mercury in a closed tube we did not find any gas above the solution. We wondered if this result were due to the gas remaining in a supersaturated condition or if there were two forms of methaemoglobin, one containing more oxygen than the other [Roaf and Smart, 1922]. Experiments were carried out to test this possibility. We prepared methaemoglobin by the action of acid during exposure to various pressures of oxygen. These solutions were run into our evacuating apparatus, and in all cases the amount of oxygen obtained was well below that liberated when oxyhaemoglobin was converted into methaemoglobin in a closed vessel and then evacuated. With high concentrations of oxygen more oxygen was observed (see Exps. 2 and 3), but the amount obtained corresponded to the amount that one would expect to find dissolved at the oxygen pressure used. In one case in which air was used above the solution the oxygen content of the air was increased by the amount that would correspond to the giving off of half the oxygen of the original oxyhaemoglobin. Therefore there is no evidence that there is a form of methaemoglobin containing more oxygen at higher oxygen pressures.

Having discussed the alternative explanations of our results we wish to draw attention to some of the difficulties of the experiments. As suggested above, too much or too little acid will give results too low because acid haematin fails to give off oxygen and oxyhaemoglobin was associated with a lower yield. Therefore only those experiments in which the right amount of acid was used gave results worth recording. Incomplete saturation of the oxyhaemoglobin will give as a result less than half of the oxygen content observed by the ferricyanide method. Bacterial action will cause a loss of oxygen, and before the use of toluene as a preservative lower results were obtained. Chemical action, apart from bacterial action, might cause some loss of oxygen, so that is another possible cause of low results. In the evacuation of the methaemoglobin solution the solution to which acid had been added was viscous and it frothed badly. This frothing was one of our difficulties, and it was less easy to get off all the oxygen than from the methaemoglobin formed by ferricyanide. We attempted to use horse corpuscles obtained through the courtesy of Dr O'Brien of Messrs Burroughs, Welcome and Company (to whom our thanks are due), but we abandoned them for bullocks' corpuscles, because the horse haemoglobin became practically solid after the action of acid. We are not certain whether there is a difference due to the species or whether it is due to the absence of calcium in the case of the horse corpuscles, which were obtained from oxalated blood and washed with oxalated saline.

In view of these various reasons why the amount of oxygen obtained might be less than the theoretical amount we consider that our results show a very good approximation to 50 % of the dissociable oxygen of oxyhaemoglobin.

Hüfner and Külz [1883] are frequently quoted as having proved that methaemoglobin contains the same amount of oxygen as oxyhaemoglobin. Their experimental method was to treat solutions of oxyhaemoglobin and methaemoglobin with urea and nitric oxide. Their results show that the same amount of nitrogen is given off in each case, but that may be due to only part of the oxygen of oxyhaemoglobin being concerned in the reaction. Küster [1910], in discussing this problem, points out that hydrocyanic acid will combine directly with methaemoglobin but not with haemoglobin. Oxyhaemoglobin reacts slowly with hydrocyanic acid. He concludes that methaemoglobin is intermediate between oxyhaemoglobin and haemoglobin.

Methaemoglobin is formed by such a variety of agents, some oxidising and some reducing [for instance palladium-hydrogen, Hoppe-Seyler, 1877, 1878], that one is led to the belief that it is the stable form in acid solution. Alkaline methaemoglobin also seems to be a stable form on the alkaline side, but it is easily converted into haemoglobin by reducing agents without passing through the form of oxyhaemoglobin (Haldane).

If the deduction made in this paper is correct the equations given by Haldane [1898] for the action of ferricyanide on haemoglobin and on oxyhaemoglobin should be rewritten with two molecules of ferricyanide instead of four. If there were a reasonable method for the estimation of ferrocyanide in the mixture this might be put to the test of experimental verification.

$$HbO_2 + 2K_3Fe(CN)_6 + 2NaHCO_3 = HbO + 2K_3NaFe(CN)_6 + 2CO_2 + H_2O + O_2,$$

and

$$Hb + 2K_3Fe(CN)_6 + 2NaHCO_3 = HbO + 2K_3NaFe(CN)_6 + 2CO_2 + H_2O.$$

Conclusions.

Oxyhaemoglobin on conversion into methaemoglobin loses half of its dissociable oxygen. This is shown in our experiments by the actual liberation of oxygen as the result of the action of acid. In Buckmaster's experiments the evidence is that hydrazine hydrate liberates only half as much nitrogen when it reduces methaemoglobin as when it reduces oxyhaemoglobin.

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LXXIV. ON GLUTATHIONE. IV. CONSTITUTION.

By JUDA HIRSCH QUASTEL, CORBET PAGE STEWART and HUBERT ERLIN TUNNICLIFFE (*Shuttleworth Student, Gonville and Caius College*).

From the Biochemical Laboratory, Cambridge.

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Glutathione was isolated from yeast and from various animal tissues by Hopkins [1921]. It is a white amorphous powder, non-hygroscopic when pure. It is very soluble in water, but insoluble in the usual organic solvents. Heated in a capillary tube, it softens at 165–167°, and melts with evolution of carbon dioxide at 182–185°. The yield of glutathione from fresh tissues was about 0·01–0·02 %; from yeast, the usual source, a similar amount was obtained.

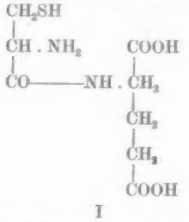
On the following evidence, Hopkins [1921] considered glutathione to be a dipeptide of glutaminic acid and cysteïne. The elementary analysis agreed with the formula $C_8H_{14}O_5N_2S$, which such a dipeptide would possess. The peptide structure was shown by the fact that the primary-amino nitrogen, as determined by the method of Van Slyke [1911], was half the total nitrogen, and was, moreover, doubled by hydrolysis. Finally, acid hydrolysis of glutathione gave *d*-glutaminic acid and *l*-cystine.

The sulphydryl group of cysteïne is readily oxidised, even by atmospheric oxygen, giving the disulphide cystine.

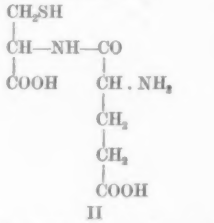
$$2\,CH_2(SH).CH(NH_2).COOH + O = COOH.CH(NH_2).CH_2.S.S.CH_2.CH(NH_2).COOH + H_2O.$$

Similarly, glutathione can be obtained in two forms: one—"reduced glutathione"—containing cysteïne: the other—"oxidised glutathione"—containing cystine. The importance of glutathione in tissue oxidation processes depends on the ready inter-convertibility of these two forms.

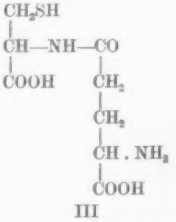
A dipeptide of glutaminic acid and cysteïne must possess a structure represented by one of the following formulae:



I



II

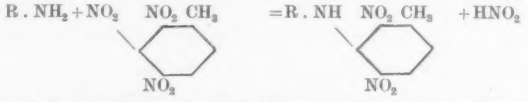


III

Our problem was therefore to find which of these three formulae is that of glutathione.

The first stage was to find through which amino group—that of glutaminic acid, or that of cysteïne—the linkage of the two amino-acids was brought about. For this purpose two methods were adopted, both of which had previously been used by Barger and Tutin [1918] in determining the constitution of carnosine.

In the first method, use was made of the power possessed by 2 . 3 . 4- and 2 . 4 . 5-trinitrotoluene, of condensing with primary amino groups.



The condensation product of glutathione and 2 . 3 . 4-trinitrotoluene, on hydrolysis, yielded free cystine. It must be concluded, therefore, that the trinitrotoluene had reacted with the amino group of the glutaminic acid part of glutathione, and that the amino group of cysteïne had formed part of the peptide linkage. From the hydrolysis mixture, a yellow substance was also isolated: this was probably the condensation product of glutaminic acid with trinitrotoluene. Its identity was not definitely ascertained however, owing to the failure of all attempts to obtain a satisfactory condensation between trinitrotoluene and free glutaminic acid.

The second method consisted in the replacement of the free primary amino group of the dipeptide by hydroxyl, and identification of the hydroxy-acid obtained after hydrolysis of the resulting compound. In this way, α-hydroxyglutaric acid was isolated, a result confirmatory of that obtained by the first method.

These experiments agree, then, in showing that in glutathione, the free primary amino group is in the glutaminic part of the molecule, and that it is the amino group of the cysteïne which is involved in the peptide linkage. Formula I, therefore, is not that of glutathione, but the evidence so far does not differentiate between Formulae II and III.

It remained, therefore, to determine which of the two carboxyl groups of glutaminic acid was, in glutathione, condensed with the amino group of cysteïne. For this purpose, use was made of an investigation by Dakin [1905], who found that α-amino-acids, oxidised by means of hydrogen peroxide in presence of a trace of iron salt, gave carboxylic acids containing one carbon atom less than the original amino-acid. The course of the reaction, he has shown, may be represented thus:

$$\mathrm{R.CHNH_2.COOH \xrightarrow{O} R.CHO + NH_3 + CO_2 \xrightarrow{O} R.COOH + NH_3 + CO_2.}$$

Glutaminic acid, oxidised in this way, yielded succinic acid [Dakin, 1908]. Formula II, is that of a γ-amino-acid, Formula III, of an α-amino-acid. A compound of Formula III, oxidised by hydrogen peroxide, would be converted to a substance containing the succinyl radicle in place of the original glutaminyl radicle. This compound would probably be, not succinyl-cysteïne,

but succinyl-cysteic acid, the sulphydryl of the cysteïne having been oxidised to the sulphonic acid group. After, and only after, hydrolysis of this compound, would free succinic acid be obtained as one of the products.

A compound of Formula II, on the other hand, must behave rather differently. Even if the $>CH.NH_2$ group be oxidised, the resulting carbonyl must, by continued oxidation, lead to a breakage of the chain, and the immediate formation of succinic acid *before* hydrolysis.

Experiments were carried out on these lines, and it was found that simple oxidation of glutathione by hydrogen peroxide produced no trace of succinic acid, whereas after hydrolysis of the oxidation product, succinic acid was isolated in a yield amounting to 50 % of the theoretical. It is therefore concluded that the structure of glutathione is represented by Formula III.

The ultimate proof of constitution lies in the synthesis, by known reactions, of the substance under consideration. Hence, until glutathione has been synthesised—a task on which the authors are at present engaged—the conclusions set forth in this paper must be regarded as being in no way final. The analytical evidence, however, which is briefly summarised below, appears to be quite strong enough to justify the deductions drawn from it.

1. Glutathione condenses with 2 . 3 . 4-trinitrotoluene, its free amino group being thereby bound. Hydrolysis of the resulting compound gives cystine, but no free glutaminic acid.

2. The free amino group of glutathione having been replaced by hydroxyl, a compound is obtained which, by hydrolysis, gives α-hydroxyglutaric acid.

These entirely different methods agree in showing that in glutathione, it is the amino group of the cysteïne which is concerned in the peptide linkage.

3. Oxidation of glutathione by hydrogen peroxide yields succinic acid only after hydrolysis of the oxidation product.

Hence glutathione is itself an α-amino-acid, and of Formulae II and III, only the latter satisfies this condition. Moreover, this result affords additional confirmation of the deductions drawn from the previous experiments, since a compound of Formula I could not yield succinic acid under the circumstances.

Owing to the low concentration in which glutathione is found, and the long and complicated method necessary for its separation, the amount available for this work was very small. The whole of the experiments described were carried out on rather less than 2 g. of material.

Experimental.

Condensation of Glutathione with 2 . 3 . 4-Trinitrotoluene.

0·3 g. of "oxidised" glutathione and 1·0 g. of 2 . 3 . 4-trinitrotoluene, dissolved in 40 cc. of 60 % ethyl alcohol, were heated under a reflux condenser, on the water-bath, for two hours. After ten minutes the liquid began to turn yellow, and the colour gradually deepened. The clear solution was evaporated to dryness on the water-bath: absolute alcohol was added and evaporated off to

remove the last traces of water. The residue was extracted three times with dry benzene, to remove unchanged trinitrotoluene. The yellow crystalline powder remaining behind was dissolved in hot water. The solution was filtered and the filtrate was evaporated to dryness on the water-bath. The yellow residue was washed with absolute alcohol, in which it was only very slightly soluble. Absence of glutathione from this residue was shown by its inability to give any precipitate with mercuric sulphate in acid solutions.

The condensation product thus obtained was a yellow semi-crystalline substance, very soluble in water, only slightly soluble in ethyl alcohol, insoluble in ether and benzene. Heated in a capillary tube, it softened at 115° and melted sharply with decomposition at 202° (uncorr.). Yield 75 % of the theoretical.

Analysis:

0·1113 g. dried at 100°; 0·1695 g. CO_2; 0·0501 g. H_2O.

Found C = 41·54 %; H = 4·98 %.

Calculated for $(C_{15}H_{17}O_9N_4S)_2$ C = 41·94 %; H = 3·97 %.

Nitrogen was estimated by a modified micro Kjeldahl [Stewart, 1923].

10·0 mg. contained 1·314 mg. Nitrogen N = 13·14 %.

Calculated for $(C_{15}H_{17}O_9N_4S)_2$ N = 13·06 %.

Hydrolysis of the Condensation Product.

0·4 g. of the condensation product were dissolved in 15 cc. of 30 % sulphuric acid and boiled under a reflux condenser for four hours. At the end of this time, the liquid was cooled, and thoroughly extracted with ether. The deep yellow ethereal extracts, on evaporation left a yellow residue, readily soluble in alcohol and in water, which was probably the condensation product of glutaminic acid and trinitrotoluene. The almost colourless aqueous layer was diluted to 60 cc. with distilled water, and excess of mercuric sulphate solution was added. The voluminous white precipitate was separated by centrifuging, and washed repeatedly with distilled water. It was then suspended in water, and decomposed by hydrogen sulphide. The filtrate from mercuric sulphide was boiled until all the hydrogen sulphide had been expelled. The colourless solution then gave the following reactions:

1. A violet coloration (when neutralised) with triketohydrindene hydrate.
2. A vivid permanganate coloration with sodium nitroprusside in presence of ammonia.
3. A black precipitate of lead sulphide on prolonged boiling with lead acetate and sodium hydroxide.

It was therefore concluded that the solution contained cysteïne.

A slight excess of baryta water was added, and after removal of the barium sulphate, the solution was aerated until the nitroprusside reaction was negative—*i.e.* until the conversion of cysteïne to cystine was complete. Barium was then quantitatively removed by addition of sulphuric acid, barium

sulphate was filtered off, and the clear liquid concentrated on the water-bath. On standing, cystine separated in typical hexagon-shaped plates, and on further concentration of the mother liquors, in acicular aggregates.

Nitrogen by micro Kjeldahl:

8·7 mg. contained ·999 mg. Nitrogen N = 11·48 %.
Calculated for $C_6H_{12}O_4N_2S_2$ N = 11·66 %.

De-amination of Glutathione.

0·5 g. of glutathione was dissolved in 10 cc. of water, and 2·0 g. of freshly prepared silver nitrite were suspended in the solution. The liquid was cooled in ice, and 20 cc. *N* HCl were added gradually with constant stirring. The mixture was left in ice for one hour, and was then allowed to stand overnight at room temperature. The precipitated silver chloride was filtered off, and the filtrate was found to be free from silver. The liquid was extracted with ether, and the dried ethereal extracts were evaporated to dryness on the water-bath. There was no residue.

Hydrolysis of De-aminated Glutathione: Isolation of α-Hydroxyglutaric Acid.

An equal volume of 35 % hydrochloric acid was added to the solution containing the de-aminated glutathione, and the mixture was boiled under a reflux condenser for four hours. After cooling, the hydrolysis mixture was thoroughly extracted with ether, and the combined ethereal extracts, dried over anhydrous sodium sulphate, were evaporated on the water-bath. There remained an oily liquid, which, on cooling, solidified to a deliquescent, crystalline mass. Yield 0·10 g. It had a fruity odour: was strongly acid, was readily oxidised by potassium permanganate, and gave no coloration with triketohydrindene. Tests for sulphur and nitrogen were completely negative. The substance melted well below 100°, but owing to its deliquescent nature, no accurate determination of the melting point could be made.

For analysis, the calcium salt was prepared by addition of calcium chloride in slight excess to the neutralised solution of the acid in slightly dilute alcohol. The precipitated salt was separated by centrifuging, washed thoroughly with alcohol and finally with ether. It was dried at 37°. A weighed quantity of the salt was transferred to a centrifuge tube, and dissolved in dilute hydrochloric acid. A slight excess of saturated ammonium oxalate was added, and the liquid was made alkaline with ammonia. It was allowed to stand one hour, and was then centrifuged. Most of the supernatant liquor (all but about 0·3 cc.) was siphoned off, and the precipitate was washed three times with 2 % ammonia by the same process. The calcium oxalate was then dissolved in 2 cc. *N* H_2SO_4, warmed on the water-bath to 60°, and titrated with 0·01 *N* potassium permanganate from a micro-burette.

6·2 mg. of calcium salt required 6·27 cc. of 0·01 *N* $KMnO_4$;
Ca = 20·22 %.
Calculated for calcium α-hydroxyglutarate, $Ca.C_5H_6O_5.\frac{1}{2}H_2O$;
Ca = 20·51 %.

Oxidation of Glutathione.

0·5 g. of "oxidised" glutathione was dissolved in a little water, and the solution was made slightly alkaline with ammonia. A trace of ferrous sulphate was added, and then 25 cc. of 5 % hydrogen peroxide. After half an hour at 70°, evolution of gas had ceased, and a further 25 cc. of 5 % hydrogen peroxide were added, the heating being continued to a total of one hour. The solution then gave no coloration with triketohydrindene, whereas glutathione itself gives a strongly positive reaction. Evidently the free amino group had been removed by the oxidation. Further, mercuric sulphate produced no precipitate. After cooling, the solution was acidified with hydrochloric acid, and thoroughly extracted with ether. The ethereal extracts, dried and evaporated, left no residue. No free succinic acid, therefore, had been formed by the oxidation.

Hydrolysis of the Oxidation Product.

The solution of the oxidation product was evaporated to dryness to remove all traces of ether and hydrogen peroxide. The residue was dissolved in 20 cc. of 30 % hydrochloric acid, and boiled under a reflux condenser for three hours. The solution was cooled and extracted with ether. The combined ethereal extracts were dried and evaporated on the water-bath, when there remained a white crystalline solid, in amount equal to 40 % of the theoretical yield of succinic acid. That this substance was succinic acid was shown in the following ways:

1. It formed bunches of thick prismatic needles, identical in form with those of succinic acid.
2. It was not readily soluble in water, and the aqueous solution was strongly acid.
3. It gave no coloration with triketohydrindene.
4. The ammonium salt, distilled with zinc dust gave the pyrrole reaction with a pine splint moistened with hydrochloric acid.
5. Heated with resorcinol and concentrated sulphuric acid, it gave the typical fluorescent solution.
6. Heated alone, it melted and sublimed in feathery needles, and produced a choking odour.
7. Its neutral solution gave a precipitate with ferric chloride solution.
8. From neutral solution, its silver salt was precipitated by the addition of silver nitrate.
9. The acid, after purification through the silver salt, melted at 180° (uncorr.) and the melting point was not altered by the admixture of pure succinic acid.

For analysis, a weighed amount of the acid, purified through the silver salt, was dissolved in water, and titrated with standard alkali from a microburette. From this neutral solution, the silver salt was precipitated, and after thorough washing was dissolved in nitric acid and titrated with standard potassium thiocyanate.

1. 15·0 mg. of the acid required 5·050 cc. 0·05*N* NaOH
[≡ 14·89 mg. succinic acid].
The silver salt required 6·3 cc. 0·04*N* KCNS
[≡ 14·87 mg. succinic acid].

2. 4·4 mg. of the acid required 1·495 cc. 0·05*N* NaOH
[≡ 4·41 mg. succinic acid].
The silver salt required 1·850 cc. 0·04*N* KCNS
[≡ 4·36 mg. succinic acid].

Our thanks are due to Prof. F. G. Hopkins, F.R.S., for the supply of material and for the kind interest he has taken in this work, also to Prof. G. Barger, F.R.S., for the specimen of 2. 3 . 4-trinitrotoluene used in this work, and for helpful criticism. Two of us (J. H. Q. and H. E. T.) are indebted to the Department of Scientific and Industrial Research for grants which have enabled us to carry out this work.

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LXXV. ON THE PRESENCE OF MALTASE IN GERMINATED AND UNGERMINATED BARLEY.

By ARTHUR ROBERT LING and DINSHAW RATTONJI NANJI.

From the Department of Biochemistry of Fermentation, University of Birmingham.

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It has long been known that when starch is hydrolysed in presence of malt diastase under certain conditions, glucose is one of the products. Some chemists have attributed this production of glucose to the presence of the enzyme, maltase, in the malt. Thus Effront in 1899, dealing with the conversion of maltose into glucose by enzyme action, remarks [1899]: "L'infusion de malt agit très peu sur le maltose, mais le malt concassé agit energiquement sur les sirops de maltose, qui se transforment en sirop de dextrose."

It is, however, on record that, when starch is hydrolysed in presence of enzyme preparations which do not contain maltase, glucose is, under certain conditions, produced. It was stated by Ling and Baker [1895] in 1895 that glucose is one of the hydrolytic products of the action on potato starch paste of the diastase prepared from kilned malt. In a subsequent paper [1897] these authors observed that they had now satisfied themselves that the production of glucose in the manner indicated was in no way connected with the kilning of the malt. However, Ling and Davis later [1902] showed that when an aqueous solution of precipitated diastase is heated at 65°, or at a few degrees above this temperature, and the solution allowed to act on starch paste, glucose is invariably present among the final products of hydrolysis. Here we have a distinct confirmation of the original observation of Ling and Baker.

Brown and Millar [1899] showed that the so-called stable dextrin can be partially hydrolysed in presence of malt diastase at 55° and that glucose is among the products. Ling [1903] showed that the malto-dextrin-α of Ling and Baker also yields about 10 % of glucose when hydrolysed in the presence of malt diastase at 55°.

Davis and Ling [1904], who measured the quantity of glucose formed from heated diastase solutions, showed that the maximum quantity of the sugar just mentioned is produced when the diastase solution is heated at 68–70°, a temperature at which maltase is inactive. Ling and Rendle [1904] showed that commercial concentrated malt extracts contain from 17–22 % of glucose. It will be seen therefore that glucose may be produced from starch and some of its hydrolytic products by diastase preparations not containing maltase.

We have indeed proved that no glucose is produced when precipitated malt diastase is used as the hydrolysing agent for starch unless the diastase has been heated to 65° in aqueous solution. Moreover, under all conditions, whether its aqueous solution has been heated or not, no glucose is produced by its action on maltose.

It appears to be otherwise with malt extract, Maquenne having recently shown [1923] that glucose is produced when maltose is treated with malt extract. As a result of our present experiments we find that maltase is present in the extract of both green and kilned malt and also in ungerminated barley. In the latter case, however, the enzyme is present in a form in which it cannot be extracted by water, for barley extract is without action on maltose whilst barley grist when added to maltose solution causes hydrolysis to glucose. Maltase is an endogenous enzyme which is very sensitive to reagents and very difficult to isolate. It is also thermo-labile, being destroyed below 70°.

The method adopted in the experiments we have carried out to demonstrate the presence of maltase was in the case of malt to act on a solution of maltose with an extract of the malt, or in the case of barley, where the enzyme cannot be extracted with water, with the barley grist. After a convenient period of incubation the percentage of maltose hydrolysed was determined by estimating gravimetrically the glucose formed as phenylosazone according to the method described by Davis and Ling [1904].

Dealing first with the malt experiments, five different kinds of malt were employed. No 1 was a green malt made from English two-rowed barley. No. 2 a green malt made from a foreign six-rowed barley, and Nos. 3, 4 and 5, were all kilned malts with diastatic powers, 23°, 52° and 102° (Lintner) respectively. The malt extract used was prepared by digesting one part of the finely ground malt with ten parts of water containing a little toluene at the ordinary temperature for 24 hours.

To 100 cc. of the filtered extracts thus obtained from the above-mentioned malts were added 2·12 g. of maltose and the solutions were incubated at 50°. A convenient volume of each of the malt extracts with a little toluene was also incubated side by side without the addition of maltose to serve as blanks. After a period of 45 hours the amount of glucose in each of the solutions was estimated gravimetrically as follows:

20 cc. of each of the solutions containing about 3 % carbohydrates were measured out into clean 25 cc. test-tubes and 1 cc. of phenylhydrazine and 1·5 cc. of 50 % acetic acid added to each. The solutions were then heated in a boiling water-bath for exactly one hour. At the end of this period the liquid was carefully decanted on to a tared Gooch crucible which was previously warmed with boiling water. Care was taken to decant the supernatant liquid on to the filter before transferring the glucosazone. The precipitate of glucosazone was then washed with 25 cc. of boiling water and then dried in a steam oven until the weight was constant. In the following results the difference in weight of the glucosazone between the blanks and actual experiments repre-

sents the amount of glucose due to the hydrolysis of maltose. The amount of glucose corresponding to the glucosazone was obtained by multiplying the weight of the glucosazone by the factor 1·98.

Table I.

Malts used	Wt. of the osazone from 20 cc. With maltose g.	Wt. of the osazone from 20 cc. Without maltose g.	Difference for 20 cc. g.	Wt. of maltose hydrolysed per 100 cc. g.	% of maltose hydrolysed
(1) English green malt	0·1990	0·1808	0·0182	0·1712	8·07
(2) Foreign green malt	0·1970	0·1596	0·0374	0·3518	16·59
(3) Kilned malt (Californian). D.P. 23°	0·2135	0·2017	0·0118	0·1110	5·23
(4) Kilned malt (English). D.P. 52°	0·3016	0·2768	0·0248	0·2332	11·00
(5) Kilned malt (Danubian). D.P. 102°	0·2818	0·2675	0·0141	0·1310	6·18

These results show conclusively that a soluble maltase is present in malts made under different conditions. The power of the enzyme appears however not to vary directly with the diastatic power of the malt expressed on the Lintner scale, but according to the temperature and rate at which the moisture has been expelled on the kiln. To account for the presence of a soluble maltase three possibilities suggested themselves.

The enzyme may exist in the barley before germination; or it may be elaborated during the processes of germination; or it may exist in the barley in an insoluble form before germination by which it may be rendered soluble. Further experiments were carried out to settle these points and the results show that the last view is the most probable. In the barley experiments starch paste was used as the substrate instead of maltose as it is known that barley diastase does not give rise to any other sugar than maltose [cp. Baker, 1902]. Our own experience, moreover, indicates that no maltase is present in barley extract. Accordingly, barley grist was employed for converting the starch paste and under these circumstances a considerable amount of glucose was formed.

The experiments were carried out as follows. To 20 cc. of 2–3 % starch paste 3 g. of the finely ground barley grist were added and the mixture with the addition of a little toluene incubated at 50° for 24 hours. At the end of this period the liquid was filtered and the glucose estimated as before in 20 cc. portions of the filtrate. Blank experiments were also carried out without the addition of starch, but no glucosazone was obtained from these.

In the following table are given the results of five such experiments.

Table II.

Specific gravity of the conversion liquid	Wts. of the osazone from 20 cc. g. With starch	Wts. of the osazone from 20 cc. g. Blank	Difference g.	Glucose per 100 cc. g.	% of maltose hydrolysed on the total solids
1008·65	0·015	Nil	0·015	0·1485	6·41
1010·30	0·053	,,	0·053	0·5248	19·00
1008·10	0·020	,,	0·020	0·1980	9·13
1007·50	0·012	,,	0·012	0·1188	5·94
1009·90	0·022	,,	0·022	0·2178	8·24

From these experiments we can safely infer that there is an insoluble maltase present in the raw barley, and that this undergoes modification during germination and is rendered partially soluble. The soluble maltase content of malt varies according as it is low dried or high dried. Green malts especially contain considerably more maltase, as the enzyme is probably partially destroyed during the kilning process.

Conclusions.

1. It is shown that malts, whether green or kilned, obtained by germinating barley contain an enzyme capable of hydrolysing maltose. The power of the enzyme depends, *caeteris paribus*, on the temperature and the way in which the malt has been heated on the kiln.

2. Diastase preparations obtained by precipitating a cold water extract of malt with alcohol do not contain maltase, as this enzyme is destroyed by the alcohol.

3. Ungerminated barley contains an enzyme capable of converting maltose into glucose. It cannot be extracted with water, but its activity is demonstrated by allowing ground barley (grist) to act on maltose.

Our thanks are due to W. J. Harper (Assistant in the Department) for carrying out some of the analyses connected with this work.

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LXXVI. THE INFLUENCE OF THE ANTENATAL FEEDING OF PARENT RATS UPON THE NUMBER, WEIGHT AND COMPOSITION OF THE YOUNG AT BIRTH.

By VLADIMIR KORENCHEVSKY and MARJORIE CARR.

From the Department of Experimental Pathology, Lister Institute.

(*Received July 13th, 1923.*)

In our previous experiments [1923, 2], as well as in the experiments of Korenchevsky [1921, 1922], we came to the conclusion that, as the result of a properly constituted diet taken by the mother during pregnancy or lactation, rickets and general disorders of nutrition in the offspring can be mitigated or even prevented for a considerable time; or *vice versa*, a diet, improperly constituted, may cause or aggravate serious nutritional diseases in her young. These facts were proved by experiments on young rats immediately after weaning and also when about 2–3 months old.

In the present investigation we studied the following questions:

1. Has the mother's diet before and during pregnancy any influence on the weight and number of the young, as well as on their chemical composition, at the time of birth?

2. Has the father's diet before conception any influence upon the same factors?

3. To what cause were due the striking differences found after weaning [Korenchevsky, 1921, 1922; Korenchevsky and Carr, 1923, 2] in the composition of the skeleton of the young born of mothers on normal diets. For instance, is the storage of calcium and phosphorus in the foetuses increased when the mother is on a diet rich in these elements and in cod-liver oil?

The experiments were conducted on 30 litters of rats. The technique of the experiments and the diets used were described in Korenchevsky's previous papers [1921, 1923]. The mother rats were fed on the following basal diets [for details see Korenchevsky, 1922, 3, p. 7]:

D 1 consisting of milk, cabbage, oats, bran and white bread.

N 2, containing a liberal amount of fat-soluble factor in the form of butter and cod-liver oil, 0·52 % Ca and 0·5 % P (in fresh diet).

$-A$, a diet similar to *N* 2, but deficient in fat-soluble factor, the only source of fat being cotton seed oil, and containing only 0·25 % Ca in the fresh diet.

In each series of experiments the mother rats were taken from the same litter and the father rats from another litter. After weaning, the young rats were put on special diets: some were put on *N* 2 diet and were kept on this the whole time. Rats are usually sterile on $-A$ diet, therefore, before conception the mother rats in this group received *D* 1 diet and only from the day of conception or about one week before this, were put on $-A$ diet on which they were kept during the whole pregnancy. The father rats in this group were also kept on *D* 1 diet and about a fortnight before conception were put on $-A$ diet.

The experiments were so arranged that the mothers and fathers in each series were approximately of the same age at the day of conception. The pregnant rats were carefully watched during the last period of pregnancy and immediately after birth, or in some cases 1–2 hours later, the young were killed by breaking their necks. After the stomachs and intestines had been removed the young were weighed and used for estimation of water, calcium (McCrudden's method), phosphorus (Neumann's method) and nitrogen (Kjeldahl's method).

The results of our experiments are seen from Tables I and II. The different diets of father and mother rats are clear from Table II. The number, weight and water content of the young were estimated in all 30 litters, but only in 13 of these were the total calcium, phosphorus and nitrogen determined.

Table I. *Average Weights of Mothers.*

Diets of mothers		Weight of mothers	
Before conception	During pregnancy	Four weeks before birth	After birth
N 2	*N* 2	179	212
D 1	$-A$ or *D* 1	149	171

Table II. *Average Number, Weight and Composition of the Young at Birth.*

No. of group	Diets: Of father	Diets: Of mother: Before conception	Diets: Of mother: During pregnancy	Number of litters	Average number of young in each litter†	Average weight*: Of the whole litter	Average weight*: Of one fresh young	Average weight*: Of one dry young	In young: H_2O %	In young: Ca % Fresh	In young: Ca % Dry	In young: P % Fresh	In young: P % Dry	In young: N % Fresh	In young: N % Dry
I	*N* 2	*N* 2	*N* 2	10	10	47·00	4·71	0·64	86·31	0·31	2·26	0·30	2·12	1·60	11·70
II	*D* 1 or $-A$	*N* 2	*N* 2	4	11	47·92	4·90	0·68	86·32	0·30	2·19	0·32	2·34	1·65	12·06
III	*D* 1 or $-A$	*D* 1	$-A$	12	8	35·06	4·64	0·66	85·97	0·35	2·49	0·35	2·49	1·73	12·33
IV	*N* 2	*D* 1	*D* 1 or $-A$	4	8	37·82	4·70	0·66	86·24	0·31	2·25	0·30	2·18	1·65	11·99

* The figures in these three columns represent the weights of the young after removal of stomach and intestines.

† The figures in this column are given to the nearest whole number.

The results of our experiments can be summarised as follows (see also Tables I and II):

1. The diet of the father before conception had no influence whatever over the young, either on their number, weight or composition, at birth.

2. The diet of the mother before conception and during pregnancy has influenced the litter in some respects: the mothers kept on normal diet containing an excess of fat-soluble factor and calcium bore litters larger in number

and total weight than those born of mothers fed on usual or fat-soluble-deficient diets.

3. The larger total weight of the litters was due to the greater number of the offspring in each litter and not to the increased weight of each individual.

4. A large number of young were born dead in those litters, whose mothers were fed on deficient diets: in 4 litters from well-fed mothers only in one litter was one young rat found dead; in 16 litters from the mothers kept on a deficient diet, 1, 3, 5 and 7 dead young respectively were found in 4 litters.

5. At the day of birth the H_2O, Ca, P and N content in the young was nearly the same irrespective of the mothers' previous diet. A slightly better composition of young was found in some of those (see Group III in Table II) from parents on the deficient diets.

6. The striking influence previously shown by Korenchevsky [1921, 1922], Korenchevsky and Carr [1923] of different diets of mother rats shown in the skeleton of their young 24–85 days after birth cannot therefore be due to a variation in the reserve of P and Ca in the foetuses and is probably due to the different degree of storage of fat-soluble factor in the foetuses.

7. The normal chemical composition shown to exist in foetuses born of mothers kept on a diet deficient in fat-soluble factor is in accord with the accepted belief that the maternal organism will as far as possible yield all the necessary substances to her offspring even by the sacrifice of her own tissues. However, in our experiments, the storage of these substances, though being sufficient at birth, did not suffice in the post-natal life of the offspring.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We wish also to express our sincere gratitude to Professor C. J. Martin for his continuous support in this investigation.

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LXXVII. ON THE RELATIONS OF THE PHENOLS AND THEIR DERIVATIVES TO PROTEINS. A CONTRIBUTION TO OUR KNOWLEDGE OF THE MECHANISM OF DISINFECTION.

PART IV. THE HALOGEN PHENOLS.

By EVELYN ASHLEY COOPER and DENNIS LEYTON WOODHOUSE.

From the University of Birmingham.

(*Received July 6th, 1923.*)

There are in constant use a large number of disinfectants which have proved of great service for purposes of external disinfection under various circumstances, but so far the parallel problem of internal disinfection has received no adequate solution. For success in this direction a wider knowledge would appear to be necessary on the following subjects:

1. The evaluation of the effects of introducing specific groupings in various positions in organic compounds on their physiological and germicidal activity.
2. The constitution of proteins and other essential constituents of living cells and the chemical and physical action of poisons thereon.

A certain amount of research has been conducted in each of these branches, perhaps most particularly in the case of the first. Among other groups the introduction of halogens in various organic compounds was investigated by Erlich and Bechhold [1906], and the general conclusion that the introduction of halogens increased bactericidal power was reached. The present investigations, while not directly probing the intricacies of protoplasmic composition, yet have a bearing on the above problems, and information concerning the following questions has been obtained:

1. The effects on germicidal power of the introduction of halogens in different positions in the aromatic nucleus and side chains.
2. The mechanism of disinfection in the case of the phenol and chlorophenol series, as revealed by the correlation of their germicidal efficacies with their action on various proteins and other protoplasmic constituents.
3. The possibility of the discovery of substances that would find application as internal disinfectants.

Halogen Phenols.

In the first place, the bactericidal powers upon different micro-organisms of the *isomeric chlorophenols*, and certain other halogen-phenols, have been compared.

Germicidal power was determined by two methods.

1. Chick-Martin [1908] method (in absence of organic matter).
2. Inhibitory method in peptone-broth [Morgan, Cooper and Burtt, 1923].

Table I. *Chick-Martin Method.*

Phenol coefficients at 20°. Period of disinfection 30 minutes

Organisms	Chlorophenols			Bromophenol
	o	*m*	*p*	*p*
B. coli comm. (1)	3·4	5·2	5·0	7·4
" " (2)	4·1	5·6	—	—
B. typhosus	3·7	4·8	4·7	8·0
B. pyocyaneus	3·6	—	—	—
Staph. py. aur.	4·2	5·0	5·0	—
Strept. haem.	3·0	4·6	3·8	—

The results show that:

1. *m*-Chlorophenol is slightly more efficacious than *p*-chlorophenol, which in turn is more active than the *o*-isomeride. Position isomerism is thus an important factor determining bactericidal power.

2. Slight differences are observed in the relative bactericidal powers of the isomeric chlorophenols when tested with different organisms, but the selective action is not very marked.

3. *p*-Bromophenol is more effective than *p*-chlorophenol.

By the inhibitory test (48 hours at 37° in peptone-broth) coefficients of about the same value were obtained, indicating that the relative germicidal powers of phenol and its halogen derivatives were not appreciably affected by presence of organic matter, rise in temperature, or longer period of disinfection.

Tests by this method in nutrient agar media were also made with the acid-fast organism *Smegma*, and, as before, *p*-chlorophenol was more potent than the *o*-compound.

Halogen Quinols.

It was found that the solubilities of the series of chloroquinols were such as might afford a unique series for investigating the effect of continued halogen substitution, especially as the second hydroxyl group in *para*-position to the hydroxyl in phenol does not measurably affect germicidal action (Table II).

The tri- and tetra-bromoquinols, however, were insufficiently soluble, so this series is restricted to the mono- and di-bromo-compounds.

The bactericidal powers of the various quinols are given in the following table:

Table II.

	Phenol coefficients		
	B. coli	*B. typhosus*	*Staph. py. aur.*
Quinol	0·96	1·0	1·1
Mono-chloroquinol	5·8	6·0	5·2
Di-chloroquinol	12·7	12·6	—
Tri-chloroquinol	23·0	24·0	—
Tetra-chloroquinol	34·0	35·0	—
Mono-bromoquinol	11·4	—	—
Di-bromoquinol	13·8	—	—

It is seen that all the halogen quinols are much more active than either quinol or phenol, and that germicidal power invariably increases as substitution

of halogen continues. The entrance of the first halogen atom, however, has the greatest effect, and the increment in bactericidal power gradually becomes less with further substitution. As in the case of the phenol derivatives, the bromoquinols are more efficacious than the chloro-compounds, but the germicidal power of the bromo-derivatives appears more readily to approach a limiting value, with the result that dibromoquinol is only slightly more active than dichloroquinol. It is unfortunate that the tri- and tetrabromoquinols are insoluble.

Monochloroquinol may be regarded as *o*- or *m*-chlorophenol, in which a second hydroxyl group has been substituted. As a general rule the introduction of *OH* groups into organic substances tends to diminish germicidal power, but in the case of chloroquinol there is an exception to this generalisation, as it is actually more efficacious than either *o*- or *m*-chlorophenol.

It was previously found that the bactericidal and inhibitory powers of the chlorophenols were much of the same order. The following results, however, show that this is not the case with the chloroquinols.

Table III.

	Inhibitory phenol coefficients *B. coli*, 48 hours at 37°
Di-chloroquinol	10
Tri-chloroquinol	7
Tetra-chloroquinol	6

A comparison with Table II shows that the inhibitory coefficients are less than the germicidal figures, and furthermore, that while the latter increase with the number of halogen atoms, the former diminish in magnitude. It is significant that the chloroquinols caused the production of red colorations in the peptone broth, and it would appear that they became oxidised to chloroquinones, which were then put out of action by the peptone and other nitrogenous constituents.

In marked contrast are the results obtained with *p*-nitrosophenol and allied compounds, the bactericidal and inhibitory powers of which are set out in Table IV:

Table IV

	Organism: *B. coli*	
Substance	Phenol coefficient 30 mins. 20°	Inhibitory phenol coefficient 48 hrs. 37°
p-Chlorophenol	5·0	5·0
p-Nitrosophenol	1·1	30·0
Aniline	0·5	0·5
p-Nitrosoaniline	—	340
p-Nitrosodimethylaniline	50	540

It is seen that while *p*-nitrosophenol is approximately equal to phenol and less efficacious than *p*-chlorophenol in actual germicidal power, yet it is considerably more active than either as an inhibitory agent. *p*-Nitrosophenol thus not only retains its activity in peptone broth, but becomes much more

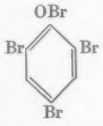
effective when the period of disinfection is lengthened and temperature raised to 37°. This is also the case with *p*-nitrosodimethylaniline, which is actually inhibitory in a concentration of 1 in 300,000. It is remarkable, furthermore, that while aniline is weaker than phenol as an inhibitor, *p*-nitrosoaniline is ten times more efficacious than *p*-nitrosophenol, and the substitution of methyl groups in the amino-group leads to a further enhancement in activity. The inhibitory power of these nitroso-compounds is destroyed by the presence of either serum or urine, so that it is unlikely that they will be of any value as internal disinfectants. Theoretically, however, the study of these compounds is of great interest, as aqueous solutions of nitrosoaniline and its dimethyl-derivative are decolorised by proteins, *e.g.* gelatin or egg-albumin, while with urine they give a brown coloration, and they thus seem to react chemically with proteins and allied substances.

Practical Application of the Halogen Phenols.

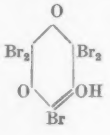
Erlich and Bechhold [1906] found there was insufficient margin between the bactericidal and toxic doses of the chlorophenols to enable these substances to be used for purposes of inner disinfection. Furthermore, they are known to be unstable in the animal organism, being converted into inactive sulphuric and glycuronic acid derivatives.

We therefore thought that it would be of practical interest to attempt to prepare derivatives of the phenols which are stabilised by the substitution of the hydrogen of the hydroxyl group. Experiments were therefore devised to replace the hydroxylic hydrogen by halogen.

Tribromophenol bromide



was first prepared by the action of excess of bromine upon phenol, and dichlorobromophenol bromide was prepared by the similar action of bromine upon the corresponding halogen phenol. These compounds are insoluble in alkali, liberate iodine from KI, and are probably tautomeric. They were, however, too insoluble in water to be of value as germicides, saturated solutions having no perceptible inhibitory power. A more promising water-soluble substance was next prepared by the action of bromine on phloroglucinol, viz. pentabromodiketoxy-*cyclo*-hexenol.



The phenol-coefficient by the Chick-Martin method was 50 (*B. coli*), but, owing to the reactivity of certain of the bromine atoms, the substance failed in the presence of organic matter, and its inhibitory coefficient was $< 1{\cdot}5$ with *Staphylococcus* and *B. typhosus*.

The Action of Halogen Phenols on Proteins.

Although the chemical nature of the cell constituents of even the most familiar bacteria has only been investigated in an incomplete and fragmentary way, it is generally believed that their life functions are intimately associated with their protein content. It is therefore not illogical to suppose that information concerning the action of some chemical substances, having well pronounced germicidal properties, upon a variety of well-known proteins, might throw light upon the mechanism of the disinfecting process in cases where hitherto an explanation on a basis of chemical interaction, such as is possible with formaldehyde or sulphur dioxide, has not been regarded as probable. The best known of these so-called "non-chemical" germicides is phenol itself, which causes a very distinctive change in the nature of many proteins. Indeed the coagulating action of alcohol and phenolic compounds has been connected with their disinfectant rôle for some years. Moreover, analogies between coagulation produced by heat and by alcohol have been drawn by many workers; *e.g.* Spiro [1904], Pauli and Handuski [1909], Lewis and Mellanby [1910] and Lepeschkin [1923] are among contributors to our knowledge of this subject.

From experiments with phenol and *m*-cresol on gelatin, egg-albumin, egg-globulin, etc. [Cooper, 1912], the following conclusions were drawn as to the rôle played by these substances as germicidal agents.

1. The absorption of phenols by bacteria is only the initial stage in the process of disinfection.

2. The germicidal action which follows the absorption does not seem to be the result of a chemical union between the phenols and the bacterial proteins, but is apparently connected with the de-emulsification of the colloidal suspension.

3. The germicidal action appears to be similar in mechanism to that of heat.

4. The superiority of *m*-cresol to phenol as a germicide appears to be due to the fact that cresol precipitates proteins in lower concentrations than phenol.

By accurate analyses of phenol and *m*-cresol solutions after standing for suitable periods in contact with proteins in a manner described, figures for the partition of the disinfectants between the water and the protein phases were obtained. When certain concentrations of phenols were reached in the aqueous solutions of the disinfectants containing emulsified protein, the latter was precipitated and a low partition-coefficient $\left(\frac{\text{wt. of germicide in 1 g. protein}}{\text{wt. of germicide of 1 g. water}}\right)$

of about 3·0 was exchanged for a higher figure of about 10·0, which rose as the concentrations of the initial solutions were increased.

Thus in the curves of emulsoids showing the percentages of phenol in the protein plotted against the final concentrations in the water phase an initial straight line came to a termination at about 1·0 % of phenol in the case of egg-albumin, and at higher concentrations was continued at a much greater slope. The inversion point denoted the percentage of phenol which produced the changed condition in the protein, but no explanation was obtained as to the inner mechanism or the significance of the change in partition-coefficient.

The following experiments dealing with the action of phenol, *ortho-*, *meta-* and *para*-chlorophenols upon proteins, were designed to continue the above researches, and to connect quantitatively as far as possible the germicidal and physico-chemical evidence. In this way it was hoped to elucidate the problems raised in the work alluded to, and to correlate the structure of the above compounds, with their action on proteins and bacteria, and thus to explain the well-known specific effect of combined halogen of increasing germicidal power.

In many cases the results with phenol recorded in the papers cited have been verified, and these confirmatory experiments are included in the following for the sake of comparison with the analogous chlorophenol experiments.

Preliminary Tests.

Gelatin was submitted to the action of solutions of phenol and the three chlorophenols. With phenol no effect was noticed below 2·6 %, but in the case of the halogen compounds, distinct opalescence was discernible in concentrations as low as 0·4 %. In the case of egg-albumin, while 0·5 % phenol was necessary to cause a turbidity, 0·1 % solutions of the chlorophenols were sufficient to induce this change. This showed clearly that the chlorophenols were much stronger precipitants than phenol itself. The experiments also indicated that egg-albumin was more sensitive to the action of chlorophenols than gelatin, as had previously been shown in the case of phenol [Cooper, 1912].

This technique was not sufficiently precise for a quantitative comparison, so that further refinements indicated below were next introduced.

Quantitative Comparison of the Precipitating Action of Phenol and Chlorophenols on Egg-Albumin and Edestin.

In the experiments described in this section, the action of the phenols was allowed to take place at the iso-electric point, and the minimum concentrations required to bring about complete precipitation were ascertained.

Solutions of egg-albumin were treated with the requisite volumes of hydrochloric acid, and phenol solutions. After standing 1 hour, the solutions were filtered from the coagulum and the filtrates tested for protein. In this way

the point of complete precipitation could be ascertained, and the following results were obtained:

Table V.

Concentration of egg-albumin	0·33 %
Acid required to adjust to iso-electric point	*N*/1245 HCl
Total volume of solutions	15 cc.

Minimum concentrations inducing complete precipitation.

	%	Ratios
Phenol	1·45	1
o-Chlorophenol	0·57	2·5
m-Chlorophenol	0·34	4·3
p-Chlorophenol	0·34	4·3

It is thus seen that *m*- and *p*-chlorophenols are much more efficacious as protein-precipitants than *o*-chlorophenol, which in turn is more powerful than phenol. It would appear that the *m*-isomeride is rather more efficient than the *p*-compound, as the protein precipitate was always more definitely clumped in the former case.

Some similar experiments were next carried out with edestin. This protein was employed in the form of a solution in 7 % sodium chloride.

Table VI.

Minimum concentrations inducing complete precipitation.

	%	Ratios
Phenol	1·15	1
o-Chlorophenol	0·22	5·2
p-Chlorophenol	0·21	5·5

In the case of edestin it is thus seen that *o*-chlorophenol is still more active as a precipitant than phenol, and closely approaches *p*-chlorophenol in precipitating power. A comparison of the above results with the bacteriological data in Table I indicates an interesting parallel between protein-precipitating and bactericidal power. It is seen also that an isomeride not only exercises a selective action on a particular micro-organism, but may also act selectively as a precipitant upon an individual protein.

Determination of Partition-Coefficients of Phenol and Chlorophenols between Water and Proteins.

These estimations were carried out to ascertain to what extent the bactericidal and protein-precipitating powers of the above phenols were determined by their relative solubilities in water and proteins. The previous work had shown that, while the partition-coefficients for phenol and *m*-cresol in the case of emulsoid proteins were almost identical, *m*-cresol was distinctly a more powerful precipitant and germicide. In view of these results it was of great interest to extend the investigations to the chlorophenols. In the first place it was necessary to work out an accurate method for estimating chlorophenols, and for this purpose Lloyd's method [1905] was used. Preliminary experiments showed that with *o*- and *p*-chlorophenol accurate results could be obtained, two bromine atoms being quantitatively introduced in each case. *m*-Chlorophenol could not be accurately determined by this method, and thus the par-

tition-coefficients of only the *o*- and *p*-compounds have been studied. In the case of the cresols, however, it is interesting to note that it was the *m*-isomeride alone that was amenable to accurate estimation [Cooper, 1912].

The partition-coefficients were determined by methods already described [Cooper, 1912], gelatin, emulsoid egg-albumin, and coagula being employed. In the case of the experiments with the emulsoids, a dialyser was employed to permit of the analysis of a protein-free water-phase.

1. *Gelatin.*

(*a*) The previous experimental work was first repeated and confirmed in the case of phenol.

Table VII.

Concentrations of phenol in water-phase	Partition-coefficient $\frac{\text{Gelatin}}{\text{Water}}$
0·4–2·6 %	3·0 (average)
2·6–4·0 %	5·0–15·8 (gradually ascending with increasing concentration)

The change in partition-coefficient at 2·6 % was associated with a precipitation of the protein. Reversibility was complete, as by dilution of the water-phase the protein assumed its normal appearance and the coefficient returned to 3.

(*b*) Similar experiments were then carried out with *o*- and *p*-chlorophenol, and the results are as follows:

Table VIII.

Initial concentration in water-phase %	Condition of gelatin	Partition-coefficient
	1. *o*-Chlorophenol	
0·2–0·4	Normal	3·2
0·7–1·0	Partly precipitated	5–7
1·1–2·0	Complete precipitation	10–21
	2. *p*-Chlorophenol	
0·2–0·8	Normal	3·3
1·0	Partly precipitated	8·0
1·5–2·0	Complete precipitation	14–23

2. *Egg-Albumin; Emulsoid Condition.*

Table IX.

	Initial concentration in water-phase %	Condition of protein	Partition-coefficient
1. Phenol	0·1 –0·5	Normal	3·1
	1·0 –5·0	Precipitated	8·7–13·9
2. *o*-Chlorophenol	0·25	Normal	3·5
	0·50	Slight precipitation	4·3
	0·75–1·0	Partial „	6·7–13·5
	1·5 –2·0	Complete „	15·0–29·1
3. *p*-Chlorophenol	0·20–0·31	Normal	5·0
	0·47	Partial precipitation	6·1
	0·75–1·8	Complete „	11·7–29·5

From these experiments the following conclusions have been drawn:

1. The partition-coefficients for phenol and the chlorophenols in the case of normal gelatin and emulsoid egg-albumin are with one exception of the same magnitude, 3·0 approx. Only in the case of *p*-chlorophenol with egg-albumin is the figure somewhat higher, 5·0.

The coefficient for *m*-cresol was also found to be of the order of 3 [Cooper, 1912].

It is thus seen that in the case of various phenols with entirely different protein-precipitating and bactericidal powers, the relative solubilities in proteins and water are of the same order of magnitude, similar results being obtained with proteins widely divergent in character. These results confirm the original conclusion that the solubility of the phenols in proteins does not account for the differences observed in the precipitating and germicidal efficacies.

2. Coagulation of the proteins by the various phenols is invariably accompanied by a very considerable increase in the value of the distribution-coefficients, and this change is induced at different concentrations according to the precipitating power of the phenol.

3. The precipitation of gelatin is in every case reversible, and the reversion is accompanied by a return to the normal partition-coefficient.

4. The precipitation of egg-albumin is always irreversible, and the high coefficients are maintained after dilution of the water-phase.

3. *Egg-Albumin; Heat Coagulum.*

Table X.

	Phenol concentrations %	Partition-coefficients
Phenol	0·1–4·3	10·0
o-Chlorophenol	0·1–2·5	28·8
p-Chlorophenol	0·1–2·5	36·0

In all three cases, the protein being in the same condition throughout, the partition-law was strictly complied with, the amount of phenol dissolved in the protein being always directly proportional to the concentration. The coefficients were also all consistently higher than those obtained with the emulsoid protein.

It is of interest, furthermore, to note that, while with the gel and emulsoid proteins, the partition-coefficients bore no relation to the protein-precipitating and bactericidal powers, in the case of heat-coagula there is a distinct correspondence:

	Partition-coefficients (From Table X)	Precipitating ratios (From Table V)	Bactericidal phenol coefficients (average) (From Table I)
Phenol	1·0	1·0	1·0
o-Chlorophenol	2·9	2·5	3·6
p-Chlorophenol	3·6	4·3	4·6

It is not possible to say whether this correspondence is accidental, or is of some physico-chemical significance.

THE EFFECT OF PRECIPITATION IN INCREASING THE PARTITION-COEFFICIENT.

The increased partition-coefficient consistently observed at precipitation has so far not been explained. It is well known that a protein in the emulsoid condition is combined with a considerable proportion of water, and it was thus possible that this abstraction of water from the continuous phase might render the partition-coefficients for the emulsoid condition fictitious and of no biological significance. In this case, the increased coefficient at precipitation would be caused by water-transfer from the emulsoid, and consequent dilution of the water-phase.

We have carried out many experiments to determine the rôle of water-transfer in these systems, and the results are briefly set out in this section.

(*a*) *The influence of denaturation.* When solutions of egg-albumin are heated to 100° in the presence of certain concentrations of acid or alkali, the protein is not precipitated as a coagulum, as is the case with the iso-electric solution, but the solution remains opalescent and becomes more viscous. In the process an interchange of water between the two phases of the colloidal solution occurs. It was therefore of importance to enquire whether this exchange would affect the partition-coefficient with phenol appreciably.

Egg-albumin solutions were denaturated by heating with low concentrations of alkali or acid, and the partition-coefficients with phenol determined on the normal and denaturated samples. Control experiments were put up to ascertain whether the alkali or acid themselves affected the coefficient for the normal protein. It was found that neither reaction nor denaturation perceptibly affected the value of the partition-coefficient (about 3·0). The water-transfer attendant on the process of denaturation was thus not responsible for the increased coefficient, which must therefore be associated with the second stage in coagulation, viz. the separation of protein in particulate form.

Evidence was also obtained that the low partition coefficient held when the protein was submitted to the action of strong phenol solutions, but was maintained in the disperse-condition by omitting to adjust the reaction. Some difficulty was met with in this experiment owing to interfering action of electrolytes present in the dialysing paper, but there was no doubt that the higher partition-coefficient, *e.g.* 10, only obtained when the protein actually separated out as a precipitate.

(*b*) We therefore next proceeded to ascertain whether precipitation by any means would induce this change in magnitude, and the methods employed were as follows:

Table XI.

	Partition-coefficient
1. Coagulation of heat-denaturated protein by traces of electrolytes, *e.g.* sodium citrate, 0·1 %	11·0
2. Coagulation of same by adjustment to iso-electric point by means of acid	11·0
3. Salting-out of protein with saturated NaCl:	
(i) Albumin emulsion	2·9
(ii) „ „ +saturated salt solution	7·2
(iii) „ „ +saturated salt solution +$N/20$ acetic acid (iso-electric point)	19·8
(iv) „ „ +$N/20$ acetic acid	2·4

These results show that the partition-coefficient can be increased through coagulation either by addition of a minute amount of sodium citrate, or by adjustment to the iso-electric point by acid (the amounts of electrolytes used in each case having been found to have no effect on the coefficient for normal protein).

The salting out experiment is of special interest, because in this case a large water-transfer is induced without chemical change. When albumin is treated with a saturated solution of NaCl, water passes from the protein to the continuous phase, but the partially dehydrated protein does not separate in particulate form until the reaction is adjusted to the iso-electric point. It was previously shown [Reichel, 1909] that the addition of salt increases the absorption of phenol by heat-coagulated proteins through a physical disturbance in the solubility-ratio, and it was therefore important to control this experiment very carefully.

The results show that saturated salt solution only increases the coefficient from 2·9 to 7·2, but by adjustment to the iso-electric point there is a further rise to 19·8. As the presence of salt increases the solubility of phenol in coagula (Reichel) where water-transfer is absent, it is clear that the increased partition-coefficient is associated with the electrical adjustment rather than with the act of water-transfer or "salting-out."

A consideration of all the results in fact points to the conclusion that the increase in coefficient-value has an electrical explanation, *i.e.* is associated with the discharge of protein particles at the iso-electric point, and both sets of partition-coefficients appear to be realities.

It is noteworthy that the partition-coefficients do not change as the result of denaturation effected either by heat or phenol. It would thus appear that the increased uptake of phenol at precipitation is not of biological significance, but is a sequence to denaturation only induced by the iso-electric condition. Further support of this view has been obtained by some imbibition experiments in which use was made of the well-known fact that in the presence of small concentrations of acid, gelatin swells to an amount greatly exceeding its usual extent in water, and takes up a corresponding large volume of water into the gel structure.

In these experiments the distribution of phenol between gelatin and water was investigated under three different conditions:

1. In the ordinary way.
2. In the presence of $N/20$ HCl.
3. The gelatin was first dissolved in warm water and allowed to set. The volume of water was then made up to that used in 1 and 2 by the addition of a phenol solution, such as to yield the same initial concentration.

The results were as follows:

Table XII.

Exp.	Phenol	Gelatin	Partition-coefficient
1	50 cc. 0·8 %	1 g.	2·9
2	,,	,,	3·2
3	,,	,,	3·4

Thus the actual distribution of the water did not measurably affect the partition-coefficient.

The Nature of the Action of Phenols on Proteins.

So far it is only known that phenols act in some way upon proteins to cause their coagulation at the iso-electric point. Experiments have therefore been devised to prove finally that phenols induce some chemical change analogous to heat-denaturation, and are not merely protein-precipitants causing coagulation under favourable conditions.

To solutions of egg-albumin were added solutions of phenol, alcohol, *m*-cresol, *o*-, *m*-, and *p*-chlorophenol, and the mixtures were dialysed until the odour of the reagents had disappeared. It was then found that on adjusting the reaction to the iso-electric point, the protein was coagulated. This shows clearly that the phenols (and also alcohol) react in some unknown way with proteins, with the result that the colloidal condition of the latter is permanently altered, and separation in particulate form will take place by discharge even after removal of the reagents. Furthermore, as showing that the protein-precipitating power of the phenols is merely an expression of their "denaturating" action, it was found that chlorophenols and cresol could effect this primary change in lower concentration than phenol itself.

It would seem that the denaturation induced by phenol is not identical with that brought about by heat. Thus, during heat coagulation, sulphuretted hydrogen is evolved, and can be detected either by lead acetate or sodium nitroprusside [Harris, 1923], but we find that these tests give negative results in the case of coagulation by phenol.

The exact nature of denaturation is at present unknown, but there is evidently a distinct parallel between denaturating and bactericidal action in the case of phenols and their halogen derivatives, the introduction of a halogen atom into phenol considerably increasing both these activities, and to approximately the same degree.

The germicidal action may be due not to an actual separation of the cell-proteins in particulate form, but to an enlargement of the colloidal units as a result of denaturation and transfer of water to the protein-phase. As a consequence, the surface area of the disperse-phase is diminished, and sufficient retardation in metabolism may take place to cause finally the death of the organism.

The Distribution of Phenol between Lecithin and Water.

It was deemed of interest to ascertain if other colloids would take up phenol analogously to the proteins studied. A solution of "dialysed iron" was found to be too weak to be useful in such work, but lecithin, which forms an emulsoid solution, was found to absorb phenol in measurable quantity. The equilibria were determined as in the case of the albumin emulsoid, 20 cc. of 2 % lecithin solution being used inside the dialyser, and 50 cc. phenol solution outside.

With strong phenol solutions acting for 24–48 hours, the lecithin emulsion was changed to a thick, oily sediment, and weaker solutions effected a partial precipitation from the true emulsoid state. Normally, the lecithin formed a stable emulsion over these periods. The coefficients and other data are given in Table XIII. The absorption coefficients appear to rise gradually with the increase in concentration and progress of the agglutination of the lipoid particles, as in the case of proteins.

Table XIII. *Lecithin and Phenol.*

20 cc. 2 % lecithin solution	50 cc. phenol solution
Phenol. Final concentration water-phase	Distribution coefficient
0·2726 %	5·1
0·2949	8·2
1·319	10·4
2·093	14·4

These results are of interest in connection with the work of Schiemann and Ishiwara [1914], who showed that the presence of lecithin diminished the bactericidal power of phenol. Evidently the phenol is readily taken up by lecithin and less is therefore available for disinfection. It is also possible that this alteration in the condition of the lecithin emulsion is associated with bactericidal action quite as much as the changes induced in emulsoid proteins, and, although it is true that proteins are fundamental constituents of living cells, yet the death of the organism may be due in reality to a "denaturation" of the whole colloidal complex of the cell.

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LXXVIII. ADSORPTION AND MECHANISM OF POISONING.

PART I. IRRITANT POISONS.

By TRILOK NATH SETH.

Chemical Laboratories, Benares Hindu University, India.

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In order that we may evaluate the effect produced upon life functions by the substances artificially ingested into the organism it is necessary, according to our present ideas, to postulate that the substances enter into some sort of a combination with the constituents of the cells, which in turn transmit the effect to the whole organism. It is evident that such an interaction depends not only upon the structure of the ingested reagent, but quite as much upon the structure of the cell-substances. From the chemical standpoint great difficulties arise in an attempt to study the effect of poisons directly upon higher organisms, because of the numerous disturbing influences, such as physiological activities and localisation of poisons in particular regions of the animal system, etc. Therefore, an attempt has been made in the present work to simplify matters as far as possible by choosing comparatively simple substances for purposes of experimentation. Of course the results thus obtained are by no means absolute, yet they throw some light on this difficult problem and they may possibly indicate the probable trend of reactions in higher organisms, under a similar set of conditions.

Moore and Roaf [1904, 1906] have shown that chloroform and other anaesthetics combine with protoplasm to form unstable compounds and they presume that the formation of such compounds is the cause of intoxication. The close relationship between intoxication and poisoning can justify our holding the above view with regard to the mechanism of poisoning also. Again, Loew [1893] assumes a direct chemical union of the protoplasm with the so-called "substituting poisons," supposing the labile amino and aldehyde groups of the protoplasm to be active in effecting such a union. Such an explanation seems to be, at least in some instances, quite plausible. For example, hydroxylamine and the hydrazines, which are well-known aldehyde-reagents, are powerful poisons, while the ketoximes in which the reaction group is bound, are not so poisonous. Among others, the work of P. Karrer [1918] on the action of toxins, may be specially mentioned, as supporting Loew's theory of chemical reaction between poisons and the affected tissues. However, in spite of the support lent to this theory by numerous workers, it has failed to explain

satisfactorily the mechanism of poisoning in a very large number of cases in which it has been established on fairly firm grounds that no chemical action takes place, and that the effects produced are due to surface action only. Erhlich, for example, maintains that during the course of his numerous experiments in this direction, he was never able to establish that there had been a chemical union between the poison and the affected part of the body. In fact, he found that the various poisons could be again extracted from the tissues by means of neutral chemically inactive solvents. However, Ehrlich [1909] himself mentions a number of toxins and poisons, *e.g.* cocaine, stovaine, and compounds of trivalent arsenic, etc., as being exceptions to his generalisations.

Thus it appears that at the present stage of our knowledge we cannot accept either Loew's or Ehrlich's theory to the entire exclusion of the other. On the other hand, we must accept both, for it appears that there are cases of simple chemical union, simple surface action, and cases involving both surface action and chemical action.

Howsoever this conflict of opinions regarding the mechanism of actions may be decided, at least it is certain that the production of an effect at any desired place in the organism necessitates such a constitution of the ingested substance as to make possible its local fixation, whether it be of a chemical or of a physical nature. In other words, "sorption" [McBain, 1919] of a poison by the tissues is necessary for the production of any physiological effects. The old notion that a poison may kill by its action through the nervous system without sorption, cannot be retained in the light of our present knowledge, and therefore a study of the sorption of poisons by the principal constituents of the body, is bound to be of some interest in the elucidation of the mechanism of poisoning.

Poisons belonging to the irritant type have been dealt with in the present work, and investigations on the other groups are in progress. By far the most important of the irritant poisons are salts of arsenic, lead, copper, antimony, and chromium, and these substances are characterised by their irritant effects upon the tissues, accompanied by well-marked nervous influences.

Apart from the chemical nature of the inorganic poisons the physical factors concerned in their solution, etc., are of great importance. The part played by such factors can be easily realised when it is considered that the specific effects of such poisons depend chiefly, if not exclusively, upon their constituting ions [Blake, 1839, 1881]. Quite in agreement with the assumption of ionic effect in these physiological studies, is the great importance of the degree of dissociation, which is very decidedly pointed out by the investigations of Dreser [1893] and Paul and Krönig [1896] upon the bactericidal action of mercury salts. A further support of this theory is provided by the comparative inactivity of cacodyl compounds as poisons (Crum Brown), in spite of their arsenic content and marked solubility. The inertness of such compounds is easily explained by the fact that the arsenic in them is bound up to an organic radical and is not present in the ionic state even in water.

Results obtained by Kahlenberg [1900] and Loeb [1897, 1898], during their investigations on the action of salts on bacteria, are also in general agreement with the ionic theory of poisoning.

On the basis of the view that sorption precedes poisoning and that poisoning, at least in the case of most inorganic salts, is ionic in nature, the present work was carried out with the object of determining the sorption of the various poisons in the different regions of the animal system with a view to elucidate the mechanism of poisoning.

Experimental.

Caseinogen, representing the class of protein substances which form the chief constituents of the animal body, was chosen as the adsorbent in the course of the present work, and a number of experiments were also performed with saliva, bile, and blood-serum. The poisons tried were lead acetate, arsenious oxide, and copper sulphate.

The general procedure followed in all the experiments is outlined below.

Aqueous solutions of each poison in six different concentrations were prepared. Equal volumes (50 cc.) of each of these solutions were pipetted out into dry stoppered bottles and then a weighed amount (1·0 g.) of caseinogen (the size of the particles being between 16–20 mesh) was introduced into each bottle. After thorough mixing the bottles were kept in a thermostat at 37° (body temperature) for a given time, after which the contents of each bottle were filtered separately through filter papers which were previously soaked with the particular poison in question, washed and dried. The amount of the poison left after sorption, was determined analytically in the filtrate and knowing the initial concentration of the poison used, the sorption of each poison per gram of the adsorbent (X/M) was calculated. The concentration of the poison in 1 cc. of each solution after sorption (volume concentration) was also determined and these results were plotted against the corresponding X/M. The results obtained with each poison are given below.

Lead Acetate.

Aqueous solutions of pure lead acetate of concentrations approximating to $N/10$, $N/15$, $N/20$, $N/30$, $N/50$ and $N/100$ were prepared and stocked after filtration. 50 cc. of the poison of each concentration was used for each experiment and the extent of sorption was determined after one, three and six hours. Lead was estimated gravimetrically as lead chromate and the results, calculated in terms of $Pb(CH_3COO)_2$, are given in Table I.

The curves drawn from the data are shown in Fig. 1. The shape of these curves indicates clearly that they are typical "Adsorption Isotherms." Further support is lent to this view by the fact that logarithms of X/M and volume concentration when plotted, produce a straight line which is so characteristic of adsorption reactions.

Table I.

Concentration of original solution (in terms of $Pb(CH_3COO)_2$ per litre)	After sorption for one hour		After sorption for three hours		After sorption for six hours	
	X/M	Volume concentration	X/M	Volume concentration	X/M	Volume concentration
15·916 g.	0·1105	0·01370	0·1464	0·01299	0·1482	0·01295
10·486	0·0824	0·00884	0·1196	0·00809	0·1263	0·00796
7·834	0·0711	0·00641	0·1121	0·00559	0·1225	0·00538
5·354	0·0694	0·00396	0·0966	0·00342	0·1121	0·00311
3·124	0·0448	0·00223	0·0596	0·00193	0·0655	0·00181
1·443	0·0242	0·00106	0·0376	0·00079	0·0351	0·00084

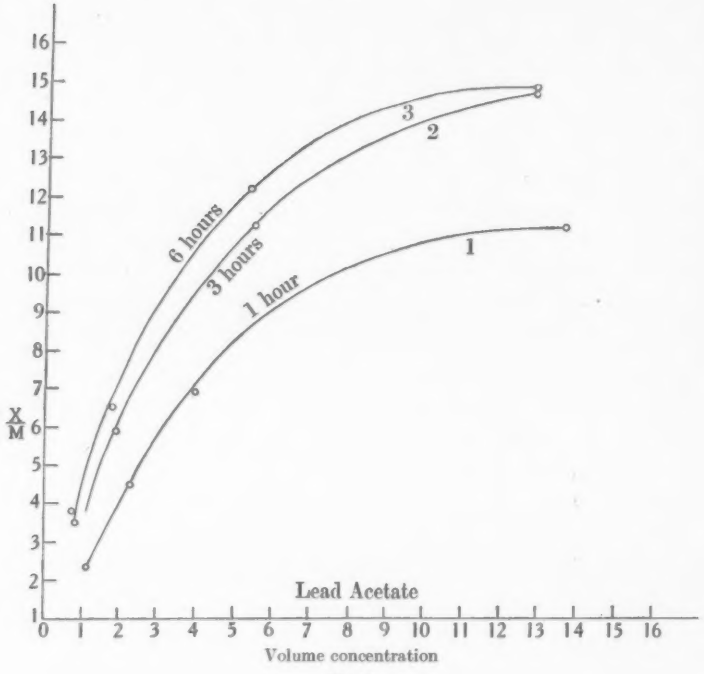

Fig. 1.

Arsenious Oxide.

Aqueous solutions of pure arsenious oxide of concentrations approximating to $N/15$, $N/20$, $N/30$, $N/50$ and $N/100$ were employed and the amount of arsenic in them was determined before and after adsorption, by titration against a standard iodine solution, in the presence of excess of sodium bicarbonate, using starch as an indicator. All other conditions of temperature, time, and procedure were the same as described before (p. 615). The results calculated, in terms of As_2O_3 are given in Table II.

Table II.

Concentration of original solution (in terms of As_2O_3 per litre)	After sorption for one hour		After sorption for three hours		After sorption for six hours	
	X/M	Volume concentration	X/M	Volume concentration	X/M	Volume concentration
3·30 g.	0·00190	0·00326	0·00220	0·00325	0·00128	0·00327
2·477	0·00107	0·00245	0·00107	0·00245	0·00092	0·00246
1·65	0·00072	0·00163	0·00072	0·00163	0·00048	0·00164
0·9895	0·00027	0·00098	0·00027	0·00098	0·00002	0·00099
0·4949	0·00022	0·00049	0·00022	0·00049	0·00012	0·00049

The nature of the equilibrium after one and three hours is distinctly different from the usual adsorption equilibrium, and what is more striking, the equilibrium conditions in the last four concentrations appear to be the same for the first three hours, after which there is a marked change, whereby the nett results of sorption after six hours, are sufficiently influenced to be thrown into the form of an "Adsorption Isotherm."

Relation between Adsorption and the Degree of Dissociation of a Poison.

It has been clearly brought out by the investigations of Dreser, and of Paul and Krönig, that substances which are dissociated to a greater extent are more powerful disinfectants and antiseptics than others which are dissociated to a lesser degree. This and other similar observations led the author to investigate the relation between adsorption and the degree of dissociation of a poison in solution. With this end in view the equivalent conductivities of the poisons at various concentrations were determined at 37°, and these results were plotted against X/M of the corresponding solutions. The curves so obtained are shown in Fig. 2. It is evident that the curves for lead acetate and arsenious oxide are both hyperbolic, and that for each concentration of the poison

$$A\,(C - P) = \text{Constant},$$

where $A = X/M$,

$C =$ Equivalent conductivity at 37°.

$P =$ a constant depending upon the nature of the poison.

It also appears that poisoning is a function of the adsorption and the degree of dissociation of any particular poison. It is further clear from Fig. 2 that with increasing concentrations of the poison the adsorption (per gram of the adsorbent) increases, while the equivalent conductivity of the corresponding solution decreases and consequently the enhanced poisonous effect due to greater adsorption of the poison is partly neutralised by the lowering of the dissociation. These facts clearly point out that there is a particular concentration at which the combined poisonous effect due to adsorption and dissociation is the maximum.

Work in this direction is in progress and the results are expected to show that merely the quantity of a poison, independent of its concentration, is not a sufficient indication of the minimum fatal dose of that poison. It is expected that there will be one particular concentration for each poison at which the

lethal dose will be most effective. On the basis of such results it may also be possible to establish certain mathematical relationships between adsorption, concentration and the lethal dose of a poison.

Copper Sulphate.

Aqueous solutions of pure Copper sulphate, of concentrations approximating to $N/1$, $N/5$, $N/10$ and $N/100$ were employed and 40 cc. of the solution of each concentration were used for each experiment. The general procedure adopted during the course of these experiments was the same as described before (p. 615), with the exception that the experiments were conducted at 28° and the extent of sorption was determined after one-fourth, one and three hours. Copper sulphate was estimated volumetrically by determining the amount of iodine liberated by it from a solution of potassium iodide. A standard solution of sodium thiosulphate was used for estimating the iodine thus liberated.

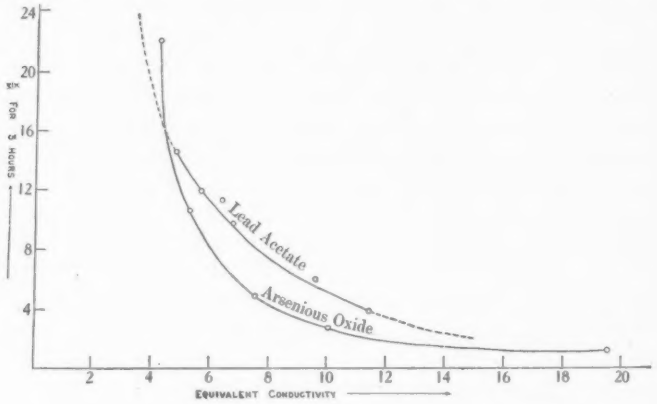

Fig. 2.

The results of the sorption of copper sulphate by caseinogen, calculated in terms of $CuSO_4 . 5H_2O$ are given in Table III.

Table III.

Concentration of original solution (in terms of $CuSO_4 . 5H_2O$ per litre)	After sorption for one-fourth hour		After sorption for one hour		After sorption for three hours	
	X/M	Volume concentration	X/M	Volume concentration	X/M	Volume concentration
122·7 g.	0·02873	0·1220	0·04357	0·1216	0·04696	0·1215
24·82	0·02572	0·0242	0·03803	0·0239	0·04473	0·0237
12·26	0·01823	0·0118	0·03202	0·0115	0·03702	0·0113
1·207	0·00549	0·0011	0·00859	0·0010	0·00875	0·0010

If curves are drawn from the above data they are seen to be typical "Adsorption Isotherms."

Copper Sulphate and Saliva.

A set of sorption experiments was conducted with approximately $N/1$, $N/5$, $N/10$ and $N/100$ solutions of copper sulphate, using human saliva as the sorbent. A few hours after meals, the salivary glands were excited by chewing some black pepper, and after thorough rinsing of the mouth with water, saliva was collected in a clean beaker. 5 cc. of this saliva were used for each experiment. All other conditions of experiment were the same as described before (p. 615). The extent of sorption of copper sulphate was determined after one-fourth, one, and twenty-four hours. The results, calculated in terms of $CuSO_4 . 5H_2O$, sorbed per gram of saliva, are given in Table IV.

Table IV.

Concentration of original solution (in terms of $CuSO_4 . 5H_2O$ per litre)	After sorption for one-fourth hour		After sorption for one hour		After sorption for twenty-four hours	
	X/M	Volume concentration	X/M	Volume concentration	X/M	Volume concentration
123·492 g.	0·00505	0·12287	0·00505	0·12287	0·00505	0·12287
24·81	0·00493	0·02420	0·00493	0·02420	0·00523	0·02417
12·26	0·00395	0·01177	0·00417	0·01174	0·00417	0·01174
1·207	0·00017	0·00119	0·00088	0·00110	0·00088	0·00110

By plotting the data given in Table IV, typical adsorption curves are produced, even in the case of saliva.

While experimenting with saliva it was observed that a precipitate was formed immediately on adding saliva to a solution of copper sulphate. Later it was found that similar observations had been recorded before in the case of other albuminous substances (*Practical Biological Chemistry*, by G. Bertrand and P. Thomas). However, it was considered worth while to study the nature of the precipitates thus obtained in order to determine their composition and the probable mechanism of their formation. With this end in view, equal amounts of saliva were added to equal volumes of copper sulphate solutions of various concentrations, and the copper-content of the precipitates thus formed, was determined after one-fourth hour and twenty-four hours. These results show that the copper-content of these precipitates is almost the same after fifteen minutes of sorption as it is after contact with the copper sulphate solution for twenty-four hours.

The facts that a precipitate is formed immediately on adding saliva to a solution of copper sulphate and that the maximum sorption takes place almost instantaneously, point to the existence of some close union between saliva and copper sulphate. Again, the amount of copper sulphate adsorbed per gram of saliva was found to vary with the concentration of the copper sulphate solution used, indicating thereby the absence of a purely chemical combination between the two substances. The only other alternative is to assume the formation of certain chemico-adsorption-compounds by the interaction of copper sulphate solution and saliva. This view is further supported

by a fact which has been clearly brought out in this work, namely, that the principal reactions between aqueous copper sulphate and saliva bear a close analogy to the phenomenon of adsorption.

Sorption of Copper Sulphate by Blood-serum and Bile.

In one set of experiments serum prepared from fresh goat's blood was used as a sorbent for copper sulphate and another set of experiments was conducted in which fresh goat's bile was employed as the sorbent. Approximately $N/1$, $N/5$, $N/10$ and $N/30$ solutions of copper sulphate were used in both the cases. 5 cc. of blood-serum or bile were employed for each experiment. 40 cc. of copper sulphate solution of each concentration were used with blood-serum, while in the case of all experiments with bile, only 20 cc. of the poison were employed each time. All other conditions of experiment were identical with those described before (p. 615). Some of the results obtained in the case of blood-serum are given in Table V.

Table V.

Concentration of original solution (in terms of $CuSO_4 . 5H_2O$ per litre)	After sorption for one-fourth hour		After sorption for twenty-four hours	
	X/M	Volume concentration	X/M	Volume concentration
124·815 g.	0·00447	0·12425	0·00594	0·12406
24·963	0·00287	0·02460	0·00402	0·02445
12·4815	0·00173	0·01226	0·00411	0·01198
4·1605	0·00039	0·00367	0·00062	0·00337

Results very similar in nature to those given in Table V were obtained in the case of bile also. Curves plotted from these data reveal the fact that bile and blood-serum behave exactly like caseinogen and saliva in the matter of adsorbing copper sulphate from its aqueous solutions.

It may be mentioned here that the phenomenon of the formation of chemico-adsorption-compounds was observed in the case of blood-serum also—a fact which was verified in a manner similar to the one employed in the case of saliva.

Conclusions.

The results of this investigation show that adsorption is the most predominant feature in the interaction between poisons like lead acetate, arsenious oxide and copper sulphate and substances of the type of caseinogen and blood-serum, etc. In a number of cases, the formation of chemico-adsorption-compounds has also been pointed out. These conclusions naturally lead one to think that the more complex phenomenon of poisoning in the living animal is also probably governed, at least in its initial stages, by laws essentially similar to the ones that guide and control the ordinary laboratory reactions of the type indicated in this paper. If the results indicated here represent, even in some degree, the trend of action of such poisons in the living organism, it is clear that adsorption forms the first step in the very complicated process of poisoning due to irritant poisons. It cannot be said at this stage whether all other

poisons act in a manner similar to the one in which irritant poisons appear to behave. Further work is necessary in this direction and it is hoped that the extended investigation may throw some more light on this obscure yet extremely interesting phenomenon of the mechanism of poisoning.

My best thanks are due to Dr S. S. Bhatnagar, for his suggestion of the problem and for the keen interest he has taken in it throughout.

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LXXIX. THE SUPPLEMENTARY VALUE OF LIGHT RAYS TO A DIET GRADED IN ITS CONTENT OF FAT-SOLUBLE ORGANIC FACTOR.

By HARRY GOLDBLATT (*Beit Memorial Research Fellow*) and KATHARINE MARJORIE SOAMES.

From the Department of Experimental Pathology, Lister Institute of Preventive Medicine.

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Introduction.

Hume [1922], and the authors [1922] showed that rats on a diet very deficient in the fat-soluble growth-promoting factor, if they are irradiated for about ten minutes daily by the mercury-vapour quartz lamp, grow much better than non-irradiated rats on the same diet. But they do not go on growing normally; eventually growth fails, and the subsequent history is that of non-irradiated rats on a $-A$ diet. This led to the conclusion that light rays promote growth by enhancing and prolonging in some way the action of the fat-soluble A which is stored in the body of animals at the time they are put on the $-A$ diet. Now some rats on the $-A$ diet employed (diet F of this investigation) develop experimental rickets [Korenchevsky, 1921; Goldblatt, 1923]. But histological examination of the bones of many irradiated rats on the same $-A$ diet revealed no evidence of rickets[1]. Most of them were practically normal, the only pathological change observed in some being slight or moderate osteoporosis. Chemical examination of the bones of these irradiated rats showed a much higher percentage of calcium than in the control rats, but lower than that of normal rats on a complete diet[1]. Recently one of the authors [Goldblatt, 1923] showed that when rats are fed on diets graded in their content of fat-soluble organic factor (from the optimal amount to practically complete absence of it) there results a corresponding gradation in the growth of the animals and in the degree of calcification of their bones. The present study was planned in order to determine the minimal amount of fat-soluble organic factor which, supplemented by light rays, can effect the normal growth of rats as well as the normal development and calcification of their bones.

[1] Unpublished.

Experimental.

In this investigation the same six diets were employed as in the study of the effect of grading the quantity of fat-soluble organic factor in a diet upon the development and calcification of the bones of rats [Goldblatt, 1923]. The basal diet, of which the six diets employed were variations, consisted of

Commercial casein	20
Starch (wheat)	50
Cotton-seed oil	15
Salt mixture (McCollum and Davis, No. 185)	5
Marmite	5
Lemon-juice	5
Distilled water	50

When the casein employed is the unpurified commercial product, it supplies to the above diet an amount of fat-soluble organic factor which will promote about 66 % of the growth of a rat on the same diet, but with sufficient cod-liver oil added to supply an optimal amount of the organic factor. The diet containing 20 % of unpurified casein as the sole source of fat-soluble organic factor will be referred to as diet *B*. An attempt was also made to determine the exact amount of fat-soluble growth-promoting factor in this diet. Rats were put on a diet completely deficient in fat-soluble *A* (diet *F* of this investigation), and when they had ceased growing for three weeks were changed to *B* diet and in addition varying amounts of cod-liver oil were administered daily. Of this oil, 2·25 mg. daily were known to promote practically normal growth for four weeks in a rat that had ceased growing on the $-A$ diet (diet *F*). When *B* diet was substituted, the addition of only 0·56 mg. of oil a day was necessary to promote the same amount of growth in four weeks. Thus the fat-soluble *A* in the remaining 1·69 mg. must have been supplied by diet *B*. A closer titration was not attempted. Thus it would seem that diet *B* contains approximately 75 % of the amount of fat-soluble *A* necessary to promote normal growth. This agrees rather well with the previous statement that diet *B* is able to promote about two-thirds of the normal growth of rats during a period of eight weeks.

In the previous investigation 3 % of crude cod-liver oil was added to diet *B*, and this resulted in a diet which contained an amount of fat-soluble organic factor considerably in excess of the optimal. This diet was called *AA* and considered the normal control diet. In the present investigation, instead of incorporating the cod-liver oil with the diet, the normal control rats were kept on *B* diet and 200 mg. of crude cod-liver oil were administered by pipette daily to the rats. This will also be referred to as diet *AA*. This is approximately half the amount of cod-liver oil which the rats on diet *AA* in the former study received, but is also far in excess of the minimal amount necessary to promote normal growth. When tested by the biological method of Zilva and Miura

[1921] 2·25 mg. daily of the oil employed gave a positive result, *i.e.* was able to promote normal growth for four weeks.

By replacing various amounts of the unpurified casein in diet *B* by casein inactivated by heat and oxidation, the amount of fat-soluble organic factor was reduced. Diets *C*, *D*, *E* and *F* were thus made, which differed from *B* only in the quantity of organic factor they contained. The following table illustrates the source and quantity of the fat-soluble organic factor in the six diets.

Table I.

	Basal diet containing		
Diet	Unpurified casein	Purified casein	Cod-liver oil
AA	20	0	200 mg. daily
B	20	0	0
C	15	5	0
D	10	10	0
E	5	15	0
F	0	20	0

Thus, considering that diet *B*, in its 20 % of unpurified casein, contains about 70 % of the minimal amount of fat-soluble *A* which will promote normal growth, it follows that diet *C* contained 52·5 %, *D* 35 %, *E* 17·5 % and *F* none at all.

Forty-eight rats (four litters of twelve[1]) were employed in this investigation. From every litter two rats were put on every one of the six diets, one being irradiated for ten minutes daily at a distance of 60 cm. by the mercury-vapour quartz lamp. Thus there were four rats on every one of the six diets (a total of twenty-four) which were irradiated daily, and the same number on the same diets who were kept in a well-ventilated room with a large double window of plate glass half an inch thick through which light had to filter. Except during the time the irradiated rats were under the lamp, they were kept in the same room as the non-irradiated controls.

Growth.

The average final weight and average maximum gain in weight of the non-irradiated rats on the six diets showed a gradation which corresponded roughly with the gradation in the fat-soluble organic factor content of the diet (see Table II). The non-irradiated rats on diet *AA* grew even slightly better than the irradiated rats on the same diet. There were two males in the former group and only one in the latter. That easily accounts for the slight difference, because males on a normal diet always grow better than females. But on the deficient diets *B*, *C*, *D*, *E* and *F* the non-irradiated rats did not grow nearly as well as the irradiated ones on the corresponding diets.

[1] The mothers of these litters were kept during pregnancy and lactation on a diet consisting of white bread, oats, maize, bran, cabbage leaf and winter milk. This diet is not very rich in fat-soluble organic factor.

Table II. *Non-irradiated Control Rats.*

No. of rat	No. of litter	Sex	Diet	Initial age days	Final age days	Days on diet	Initial weight grams	Final weight grams	Gain in weight grams	Average amount of food eaten daily grams	In bones: % H_2O	In bones: % Ca wet	In bones: % Ca dry	Histological diagnosis
G 596	XXIII	♀♂♂♀	*AA*	22	78	56	29	130	101	11·6	42·35	11·67	20·81	Normal
G 597	XXIV	♀♂♂♀	*AA*	22	78	56	19	137	118	11·6	45·93	11·28	20·86	,,
G 598	XXV	♀♂♂♀	*AA*	21	77	56	30	165	135	12·4	41·60	11·54	19·65	,,
G 599	XXVI	♀♂♂♀	*AA*	20	76	56	23	149	126	12·4	42·18	12·59	21·78	,,
						Averages	25	145	120	12·0	43·01	11·77	20·77	
G 604	XXIII	♀♂♂♀	*B*	22	78	56	27	89	62	11·5	50·06	8·43	16·87	Moderate rickets and slight osteoporosis
G 605	XXIV	♀♂♂♀	*B*	22	78	56	29	126	97	11·5	46·24	9·96	18·53	Slight rickets and slight osteoporosis
G 606	XXV	♀♂♂♀	*B*	21	77	56	36	116	80	12·8	47·00	9·35	17·63	Very slight osteomalacia
G 607	XXVI	♀♂♂♀	*B*	20	76	56	30	116	86	12·8	40·52	11·85	19·93	Nearly normal
						Averages	30·5	112	81	12·2	45·95	9·89	18·24	
G 612	XXIII	♀♂♂♀	*C*	22	78	56	27	76	49	9·1	47·50	8·67	16·50	Very slight osteomalacia
G 613	XXIV	♀♂♂♀	*C*	22	78	56	28	74	46	9·1	48·83	7·26	14·18	,, ,, rickets
G 614	XXV	♀♂♂♀	*C*	21	77	56	36	93	57	10·1	47·53	9·23	17·53	,, ,, ,,
G 615	XXVI	♀♂♂♀	*C*	20	76	56	31	88	57	10·1	46·73	8·48	15·93	,, ,, ,,
						Averages	30·5	82	52	9·6	47·64	8·41	16·03	
G 620	XXIII	♀♂♂♀	*D*	22	78	56	29	83	54	9·7	46·63	7·34	13·73	Slight rickets and slight osteoporosis
G 621	XXIV	♀♂♂♀	*D*	22	78	56	28	103	75	9·7	47·62	7·67	14·63	Very slight healing rickets and moderate osteoporosis
G 622	XXV	♀♂♂♀	*D*	21	77	56	34	88	54	9·9	41·62	10·16	17·36	Slight osteomalacia and osteoporosis
G 623	XXVI	♀♂♂♀	*D*	20	76	56	36	112	76	9·9	48·50	9·25	17·87	Very slight osteomalacia and severe osteoporosis
						Averages	32	96	64	9·8	46·05	8·60	15·89	
G 628	XXIII	♂♀♂♀	*E*	22	78	56	33	91	58	10·3	48·05	8·28	15·94	Very slight rickets and slight osteoporosis
G 629	XXIV	♂♀♂♀	*E*	22	78	56	33	68	35	10·3	46·90	7·73	14·57	,, ,,
G 630	XXV	♂♀♂♀	*E*	21	77	56	30	63	33	8·5	45·51	8·77	16·11	Very slight osteomalacia
G 631	XXVI	♂♀♂♀	*E*	20	76	56	24	54	30	8·5	49·69	6·94	13·77	Slight rickets
						Averages	30	69	39	9·4	47·53	7·93	15·09	
G 636	XXIII	♀♂♂♀	*F*	22	78	56	31	68	37	8·0	48·80	8·37	16·36	Slight rickets and moderate osteoporosis
G 637	XXIII	♀♂♂♀	*F*	22	78	56	36	51	15	8·0	44·85	8·13	14·72	Severe osteoporosis
G 638	XXIV	♀♂♂♀	*F*	22	78	56	33	72	39	8·0	50·48	6·86	13·88	Moderate rickets and osteoporosis
G 639	XXV	♀♂♂♀	*F*	21	77	56	41	111	70	9·6	43·40	9·22	16·34	Very slight healing rickets and very slight osteoporosis
G 640	XXVI	♀♂♂♀	*F*	20	76	56	28	55	27	9·6	45·73	7·50	13·84	Moderate rickets and slight osteoporosis
						Averages	34	71	37	8·64	46·65	8·10	15·02	

The irradiated rats on diets *AA*, *B* and *C* grew equally well and reached normal weights for rats of that age and breeding. The average maximum gain in weight and average final weight of the irradiated rats on diet *D* were only slightly less (7·5 %) than those on diet *AA*. But the irradiated rats on diets *E* and *F*, although they grew very much better than the non-irradiated controls on the corresponding diets, did not grow as well as those on the other four diets. Towards the end of the experimental period the growth of the irradiated rats on diets *E* and *F* had practically stopped, while the irradiated rats on the other diets were still making regular weekly gains in weight (see Table III).

Table III. *Irradiated Rats.*

No. of rat	No. of litter	Sex	Diet	Initial age days	Final age days	Days on diet	Initial weight grams	Final weight grams	Gain in weight grams	Average amount of food eaten daily grams	In bones			Histological Diagnosis
											% H_2O	% Ca wet	% Ca dry	
G 600	XXIII	♀	*AA*	22	78	56	27	129	102	12·2	43·23	12·84	22·64	Normal
G 601	XXIV	♀	*AA*	22	78	56	22	128	106	12·2	40·37	12·53	21·02	,,
G 602	XXV	♂	*AA*	21	77	56	31	168	137	11·1	43·13	11·77	20·72	,,
G 603	XXVI	♀	*AA*	20	76	56	25	109	84	11·1	46·38	11·37	21·22	,,
						Averages	26	133·5	107	11·6	43·27	12·12	21·40	
G 608	XXIII	♀	*B*	22	78	56	29	126	97	12·8	39·58	12·45	20·61	Normal
G 609	XXIV	♂	*B*	22	78	56	28	139	111	12·8	43·83	11·41	20·32	,,
G 610	XXV	♀	*B*	21	77	56	36	122	86	13·3	39·80	12·50	20·74	,,
G 611	XXVI	♂	*B*	20	76	56	29	160	131	13·3	42·02	11·73	20·29	,,
						Averages	30·5	137	106	13·0	41·30	12·02	20·49	
G 616	XXIII	♀	*C*	22	78	56	30	122	92	13·3	37·52	12·82	20·51	Normal
G 617	XXIV	♂	*C*	22	78	56	29	139	110	13·3	40·74	11·34	19·16	,,
G 618	XXV	♂	*C*	21	77	56	35	150	115	13·0	40·58	10·37	17·46	,,
G 619	XXVI	♀	*C*	20	76	56	31	122	81	13·0	39·53	12·04	19·91	,,
						Averages	31	133	99	13·1	39·59	12·06	19·86	
G 624	XXIII	♀	*D*	22	78	56	30	108	78	13·1	39·79	13·12	21·79	Normal
G 625	XXIV	♂	*D*	22	78	56	31	147	116	13·1	42·20	11·82	20·46	,,
G 626	XXV	♂	*D*	21	77	56	33	105	72	13·6	39·84	11·44	19·02	,,
G 627	XXVI	♀	*D*	20	76	56	34	133	99	13·6	38·14	12·71	20·55	,,
						Averages	32	123	91	13·3	39·99	12·27	20·45	
G 632	XXIII	♀	*E*	22	78	56	32	—	—	—	—	—	—	Severe osteoporosis
G 633	XXIV	♂	*E*	22	78	56	33	—	—	—	—	—	—	Not examined
G 634	XXV	♂	*E*	21	77	56	28	106	78	12·0	38·84	11·48	18·79	Nearly normal
G 635	XXVI	♀	*E*	20	76	56	24	108	84	12·0	40·84	11·64	19·70	,, ,,
						Averages	26	107	81	12·0	39·84	11·56	19·24	
G 641	XXIII	♀	*F*	22	78	56	31	96	65	15·1	35·55	12·66	19·67	Nearly normal
G 642	XXIV	♂	*F*	22	78	56	33	128	95	15·1	41·62	11·25	18·69	,, ,,
G 643	XXV	♂	*F*	21	77	56	38	141	103	13·3	40·63	11·26	18·97	,, ,,
G 644	XXVI	♀	*F*	20	76	56	26	105	79	13·3	43·34	11·37	20·06	,, ,,
						Averages	32	117	85	14·2	40·28	11·63	19·35	

Gross Autopsy Findings.

The irradiated rats on diets *AA*, *B*, *C* and *D* were well developed and nourished and presented no gross abnormalities of their bones. The two rats on diet *E* which died early in the experiment had acute haemorrhagic enterocolitis and their bones were thin and very brittle. The remaining two rats on diet *E* and those on diet *F* differed from those on the other four diets only in

the fact that their bones were slightly thinner and not quite as firm. One rat on diet *F* was found pregnant with eight well-developed embryos. This is interesting, since it is well known that on a $-A$ diet without irradiation rats rarely become pregnant.

The non-irradiated rats on diet *AA* were normal in every respect. Grossly the bones resembled those of the irradiated rats on diets *AA*, *B*, *C* and *D*. On diet *B* two of the non-irradiated rats showed very slight enlargement of the costo-chondral junctions and some indistinctness and irregularity of the costo-chondral line. The same occurred in two of the non-irradiated rats on diet *C* and in one of these fractures of several ribs were present. In all the non-irradiated rats on diet *D* the costo-chondral junctions were very slightly enlarged, the costo-chondral lines were not quite distinct, and the costal portion of the ribs was easily bent and broken, but no spontaneous fractures were present. On each of the diets *E* and *F* two rats had numerous spontaneous fractures of the ribs. The costo-chondral junctions were moderately enlarged in all, and the costo-chondral lines in most were very indistinct and irregular. The costal portion of the ribs and the shafts of the long bones were easily bent and broken.

Histology.

In a former communication one of the authors [Goldblatt, 1923] gave a full description with photographic illustrations of the various degrees of bone pathology which occurred in non-irradiated rats on diets similarly graded in their content of fat-soluble organic factor. The same descriptions and illustrations hold true for the diagnoses given in Tables II and III of this communication. But on every deficient diet in the present study the number of rats that developed a degree of rickets or of osteomalacia was greater than in the previous investigation (see Table I). This is undoubtedly attributable to the fact that the litters were very large and that, as was stated in the beginning, the mothers of these litters were kept on a diet not very rich in fat-soluble organic factor during pregnancy and lactation. Of the rats that were irradiated daily, not one developed the slightest sign of rickets or of osteomalacia. Of those that were on diets *AA*, *B*, *C* and *D*, the bones were normal in every respect and could not be distinguished. The bones of the irradiated rats on diets *E* and *F* were nearly normal, differing from the others only in the fact that the cortical bone was slightly thinner and the trabeculae shorter, thinner and fewer, though very well calcified. This tendency for the trabeculae to be thin was noticed in some of the irradiated rats on all of the deficient diets (*B*, *C*, *D*, *E*, and *F*).

Investigation of the Calcium Content of the Bones.

Chemical analysis for calcium content of both femora, tibiae and fibulae of all the rats was made by Aaron's method, as described in a previous communication by Goldblatt [1923]. The detailed results are given in Tables II and III, and the averages in Table IV. They show that in the non-irradiated

rats a gradation occurred in the percentage of calcium deposited in the bones which corresponded roughly with the gradation in the fat-soluble organic factor content of the diets. This is a similar result to that obtained previously [Goldblatt, 1923], but the gradation is not quite as regular. The percentage of calcium in the bones of the non-irradiated rats on diet *AA* was slightly less than that of the irradiated rats on the same diet, but the difference was very slight, being only 2·9 % less per wet and 3 % per dry weight of the bones. In a previous experiment the authors [Goldblatt and Soames, 1922] showed that rats of good stock, if kept on a perfectly normal diet in darkness, grow as well and their bones develop and calcify as well as those kept in a well-lighted room supplemented with irradiations by a mercury-vapour quartz lamp for ten minutes daily. The slight difference noted above is probably due to the fact that the rats used did not have a large pre-experimental store of fat-soluble organic factor. Rats of that kind were used expressly in this investigation in order to give a very severe test to the efficacy of the light rays.

In the bones of the irradiated rats on diets *AA*, *B*, *C* and *D*, little or no difference was found in the average percentage of calcium, it being normal in the four groups for rats of that age and breeding (see Table IV). The bones of the irradiated rats on diets *E* and *F* did not calcify quite as well as the others, though very much better than those of the non-irradiated rats on the corresponding diets.

Table IV.

	In bones of non-irradiated rats			In bones of irradiated rats		
Diet	Average % H_2O	Average % Ca wet	Average % Ca dry	Average % H_2O	Average % Ca wet	Average % Ca dry
AA	43·01	11·77	20·77	43·27	12·12	21·40
B	45·95	9·89	18·24	41·30	12·02	20·49
C	47·64	8·41	16·03	39·59	12·06	19·86
D	46·05	8·60	15·89	39·99	12·27	20·45
E	47·53	7·93	15·09	39·84	11·56	19·24
F	46·65	8·01	15·02	40·28	11·63	19·35

Summary and Conclusions.

The results of macroscopical, microscopical and chemical investigation agree in showing that rats on a diet as deficient in fat-soluble organic factor as even diet *D* of this study, can grow normally and their bones develop and calcify normally provided they are irradiated for about ten minutes daily by a mercury-vapour quartz lamp.

But the light rays cannot act as a substitute for the fat-soluble organic factor. When the latter is completely absent from the diet (diet *F*), irradiation, though it causes rats on that diet to grow much better and their bones to develop and calcify much better than non-irradiated controls, yet does not bring about absolute normality in either respect. The degree to which such rats approach the normal probably depends upon the pre-experimental store of fat-soluble organic factor which the animals possess.

When rats are used which have a poor pre-experimental store of fat-soluble organic factor, if they are not irradiated, grading the content of that factor in their diet results in a corresponding gradation of the percentage of calcium in their bones. Some of the non-irradiated rats on the diets deficient in the organic factor (*B*, *C*, *D*, *E* and *F*) develop slight to moderate degrees of osteomalacia or rickets, complicated in most instances by some degree of osteoporosis. This confirms the results obtained in another investigation by one of the authors [Goldblatt, 1923].

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LXXX. THE DISTRIBUTION OF CARNOSINE IN THE MUSCLES OF THE NORMAL AND DECEREBRATE CAT.

By T. MITSUDA.

From the Biochemical Department, University of Cambridge.

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Carnosine is noteworthy as being the only tissue constituent containing a β-amino acid. Its biological significance is as yet obscure. The work described in the present paper forms part of an endeavour to decide whether the base suffers a change of concentration during changes in the metabolic conditions of muscles.

An entirely satisfactory method for its estimation is yet lacking. Skworzow first attempted quantitative determination of carnosine by estimating the nitrogen content of the carnosine silver fraction from muscle extract. Mauthner [1913] unsuccessfully attempted the quantitative isolation of carnosine as the copper salt which had been prepared previously by Gulewitsch. Fürth and Hryntschak [1914] first attempted to found a colorimetric estimation of carnosine nitrate on the fact that histidine when treated with diazobenzene sulphanilic acid and sodium carbonate, gives rise to an intense red colour. But the results given by these authors show that the method was decidedly inaccurate. Koessler and Hanke [1919] described a colorimetric method for the estimation of iminazole derivatives based on the diazo reaction and claimed a fair degree of accuracy and facility for the method. The chief modification introduced by them was the substitution of the standard histidine solution by a standard consisting of a mixture of methyl-orange and Congo-red ("CR . MO"), which gave a colour matchable with diazotised histidine. Clifford [1921] suggested a further modification of Koessler and Hanke's method. The latter authors obtained a perfect linear relationship between the amounts of histidine diazotised and the reading of the standard (CR . MO) cylinder. Clifford, however, using standard solutions of histidine and carnosine matched against a CR . MO indicator solution found that linear relationship between depth of colour and amounts of carnosine diazotised did not hold.

On the other hand, Hunter [1921] a little later confirmed Koessler and Hanke's statements.

Hunter found, however, that solutions containing carnosine, after heating to 100° for one hour, yielded less colour than the original solution, and further that filtration through three filter papers in succession caused a loss of 44 %

of the carnosine present. He also declared there is reason to believe that part of the colour obtained is due to substances other than carnosine present in the muscle extract. Proteins had been found by Koessler and Hanke to lower the reading of a standard histidine solution by 30 %.

It is clear, then, that the colorimetric estimation of carnosine is not yet placed upon a satisfactory basis, but requires further study.

According to Clifford [1921] carnosine is absent from the flesh of all invertebrates, white and flat fishes, chelonians, and birds belonging to the finch and owl tribes, but is found in fishes rich in fat, and most reptiles and amphibians, in birds, and in all mammals. The amount present is practically constant in individuals of any one species.

After keeping the muscles of mammals and fishes under ordinary conditions for four days after death, Clifford found no decrease in amount of carnosine originally present, but putrid meat yielded smaller amounts.

The claim of Pekelharing and his co-workers that in the metabolism of Muscle Tonus there is an increase of creatine seemed to make it desirable to determine if other nitrogenous metabolites in the tissue undergo similar changes and the research here reported deals with the carnosine contents of the leg muscles of cats under normal condition and in decerebrate rigidity respectively. The determination was carried out chiefly by Clifford's modification of Koessler and Hanke's method. The author has, however, slightly modified this method as will be seen from the following description.

Method of Extraction employed.

5–10 g. of finely divided muscle were placed in an Erlenmeyer's flask of about 300 cc. capacity and extracted with 100 cc. of distilled water at 70° for 30 minutes, the extract being poured into a 500 cc. flask. The residue was ground to pulp and extraction as above was repeated three times. Finally the residue was transferred to the half-litre flask and the whole made up to about 450 cc. with distilled water. The extraction was allowed to continue for one hour at room temperature with frequent shaking, and the flask was then heated 30 minutes at 100°. After cooling, the contents of the flask were made up to the 500 cc. mark with water and then filtered. The first 20 cc. or so of the filtrate were rejected. Of the main bulk 50 cc. were placed in a dry flask and 10 cc. of a 20 % solution of metaphosphoric acid were added to precipitate the soluble protein which otherwise prevents the colour development of carnosine. The mixture was left to stand 2–24 hours.

The solution was then filtered from the precipitated protein through a dry filter paper into a dry beaker. About 20 cc. of the first filtrate were again rejected to avoid any lowering of the result due to absorption by the filter paper. 30 cc. (corresponding with 25 cc. of the original extract) were placed in a 50 cc. volumetric flask and neutralised to litmus paper with 10 % caustic soda solution and made up to the mark with distilled water. Of this filtrate 0·1–1·0 cc. was used for the colorimetric determinations. The amount thus

measured (x cc.) was diluted with $(1 - x)$ cc. of distilled water; $x/2$ cc. will be seen to represent the corresponding amount of the original extract.

Standardisation of Standard Indicator. A solution of carnosine nitrate containing 0·00001 g. of the salt in 1 cc. was used as a primary standard for the preparation of the Congo red-methyl orange solution which was employed as the permanent standard. It was diazotised in one of the vessels of a Duboscq's colorimeter. Placing $(1 - x)$ cc. of distilled water, 5 cc. of 1·1 % sodium carbonate solution and 2 cc. of diazotised sulphanilic acid solution, prepared according to Koessler and Hanke, in the test cylinder, x cc. of the standard carnosine nitrate solution was added from a burette. 8–10 minutes are sufficient to develop the maximum colour. This solution was then placed on one side of the colorimeter and the scale set at 10 mm. The CR . MO solution under preparation was then compared with it.

Different amounts of carnosine nitrate were treated as above and matched against the indicator solution. The results obtained were as follows:

	Carnosine nitrate in test cylinders	Reading of standard indicator	Theoretical reading of standard indicator
x cc.	mg.	mm.	mm.
0·3	0·03	9·6	9·6
0·4	0·04	13·0	12·7
0·5	0·05	16·5	16·0
0·6	0·06	21·8	19·2

No sharp linear relationship between the amount of carnosine and the reading of standard indicator was therefore found but the proportionality was nearer than in Clifford's experiments. From these figures which were consistently obtained the amounts of carnosine nitrate corresponding to any depth of the standard indicator between 9·6 and 21·8 can be obtained by calculation. As the molecular formula of carnosine nitrate is $C_9H_{14}O_3N_4HNO_3$, the amounts of carnosine will be obtained by multiplying the amounts of carnosine nitrate by 0·78286.

Determination of Carnosine in certain leg muscles of the Cat.

Results obtained from ten cats by the method described are shown in the following table:

Table I.

Cat No.	Gastro-cnemius	Quadriceps	Semi-membranus	Triceps	Solens	Tensor
			Carnosine %			
1	1·293	0·955	1·799	0·845	—	1·299
2	1·153	0·889	1·441	0·766	0·352	—
3	1·028	1·006	—	0·706	—	—
4	1·012	—	—	0·727	0·341	—
5	0·924	0·843	1·532	0·686	—	—
6	0·905	0·687	1·221	0·574	0·483	—
7	0·775	—	—	0·753	—	—
8	0·747	—	1·578	0·669	—	—
9	0·697	0·550	—	0·602	—	—
10	—	—	1·086	0·525	—	—

As the above table shows, markedly different amounts of carnosine are present in different muscles. The semimembranus contains the largest amounts

and the solens the smallest. These differences are consistent, in spite of the fact that the absolute amount of the base in any one muscle is seen to differ considerably when one animal is compared with another. Clifford did not find this wide variation in different animals. In regard to this point further investigation is therefore required. In favour of the reliability of the method as used by myself (at least when comparisons are in question), I would point to the close agreement between the amounts of carnosine found in the experiments now to be described. In these the muscles of opposite sides were compared in the same animal.

As stated above, the experiments were done in an endeavour to decide whether the phenomena involved in Tonus produce any change in the concentration of nitrogenous metabolites other than creatine. The particular experiments described in the present paper deal with a comparison between normal muscles and those in the condition of decerebrate rigidity. As a considerable difference exists in the amounts of carnosine in the same muscle in different cats (Table I), it was necessary to compare the amounts of the base on opposite sides of the same animal. On one side the occurrence of rigidity was prevented by cutting the motor nerve.

As a preliminary, a comparison was made of the muscles of normal cats on opposite sides (Table II). Next, also in normal cats, the effect of cutting the motor nerves on one side was investigated, estimations being made four hours after section of the nerves (Table III). Finally, in other animals, the nerves of one side were cut and decerebration then performed. Estimations were then made after the rigidity had existed for four hours (Table IV).

It is a well-known fact that the decerebrate rigidity can be seen in gastrocnemius, quadriceps and triceps. The estimation was made with these three muscles. The next three tables show the results respectively. Decerebration was carried out by Dr K. Uemo.

Table II.

Cat No.	Side	Gastrocnemius	Quadriceps	Triceps
1	Left	1·030	1·012	0·714
	Right	1·025	1·001	0·699
	Difference	0·005	0·011	0·015
2	Left	0·684	0·546	0·596
	Right	0·697	0·550	0·603
	Difference	−0·013	−0·004	−0·007
3	Left	0·769	0·696 0·533	0·753
	Right	0·775	0·690 0·525	0·740
	Difference	−0·006	0·006 0·008	0·013

Table III.

Cat No.	Side	Gastrocnemius	Quadriceps	Triceps
1	Left (nerve cut)	—	0·736	0·666
	Right (normal)	—	0·749	0·671
	Difference	—	−0·013	−0·005
2	Left (nerve cut)	—	0·832	0·667
	Right (normal)	0·924	0·843	0·689
	Difference	—	−0·011	−0·019 (−0·022)
3	Left (nerve cut)	—	0·683	0·579
	Right (normal)	0·905	0·687	0·574
	Difference	—	−0·004	0·005

Table IV.

Cat No.	Side	Gastrocnemius	Quadriceps	Triceps
1	Dec. rigidity	0·850	0·554	0·517
	Nerve cut	0·843	0·555	0·520
	Difference	0·007	−0·001	−0·003
2	Dec. rigidity	1·101	0·797	0·677
	Nerve cut	1·105	0·795	0·669
	Difference	−0·004	0·002	0·008
3	Dec. rigidity	1·300	0·961	0·849
	Nerve cut	1·296	0·950	0·840
	Difference	0·004	0·011	0·009

It will be seen on examination of the above figures that neither cutting the motor nerve nor the induction of decerebrate rigidity has any effect upon the carnosine in the muscles.

SUMMARY.

Using a colorimetric method based upon that of Koessler and Hanke, as modified by Clifford, estimations of carnosine have been made in the muscles of the cat.

The method gave highly consistent results when applied to corresponding muscles in the opposite limbs of the same animal.

The carnosine content of any one type of muscle varies considerably in different cats. Much wider differences, however, are to be observed in different muscles of the same animal, and a high or low concentration respectively would seem to be characteristic of individual muscles.

Neither section of its motor nerves nor the condition of decerebrate rigidity produces any effect upon the concentration of carnosine in muscle.

I wish to express my grateful thanks to Professor F. Gowland Hopkins for the interest he has taken in this work.

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LXXXI. THE PROPERTIES OF COLLOIDAL GUM BENZOIN.

By HEDLEY DUNCAN WRIGHT and WILLIAM OGILVY KERMACK.

From the Laboratory of the Royal College of Physicians, Edinburgh.

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Introduction.

In 1920 a new precipitation test for the cerebro-spinal fluid was introduced by Guillain, Laroche and Lechelle [1920]. This test is carried out in a manner similar to the Lange colloidal gold test [Lange, 1912]. A colloidal solution of Sumatra gum benzoin[1] is added to an equal volume of salt-solution containing varying amounts of cerebrospinal fluid. In certain of the tubes, flocculation and sedimentation of the 'benzoin' takes place, and from this, indications are said to be obtained of the nature of the disease. The zones of precipitation vary in position but more particularly in intensity, and are difficult to explain. It was with the hope of obtaining some knowledge of the mechanism of these precipitations that an investigation of the colloidal properties of 'benzoin' sol was undertaken.

Macheboeuf [1921] and Guillain, Laroche and Macheboeuf [1921] have determined that the charge on the particles in the sol is negative, but otherwise very little information is available on the subject.

In view of the fact that the behaviour of gelatin has been so elaborately studied and that this material could be obtained in relatively pure form by Loeb's [1922, 1] method this protein was first used in order to gain information as to the effect of proteins on 'benzoin.' The interesting fact was discovered, that in great dilution gelatin caused precipitation of the sol. This property, it has been found, is not peculiar to gelatin, and a similar precipitation occurs in presence of very dilute solutions of other proteins, *e.g.* haemoglobin, of the proper concentration. Neisser and Friedemann [1904] and Bechhold [1904] had shown that small amounts of gelatin rendered mastic precipitable by amounts of sodium chloride which, alone, were incapable of causing precipitation. Similar effects had also been noted by Walpole [1913] regarding the interaction of acid, gelatin and mastic sol or an emulsion of oil in water. Zsigmondy in 1901 discovered that proteins had a definitely protective action against the precipitation of solutions by electrolytes while, according to Zunz [1904], certain proteoses are, on the other hand, capable of causing precipitation. The 'benzoin' sol therefore showed itself to be somewhat remarkable

[1] Afterwards in this paper referred to as 'benzoin.'

in that it is precipitated by gelatin, without acid or salt, and, accordingly, this point has been more fully investigated. It is, however, to be noted that Gann [1917] was able to demonstrate precipitation by gelatin of gold sols which had an acid reaction but not of sols which were alkaline and considered that the hydrogen ion concentration was an important factor. In a recent summary of experimental results Pauli [1922] has stated that pure proteins, free from electrolytes, have no protective power but, on the other hand, cause precipitation of colloids.

From the study of the effect of proteins in precipitating the sol, we have been led to investigate its precipitation by electrolytes. It is well known that the power of electrolytes to cause precipitation of a colloid depends largely on the valency. This is well shown by results obtained by Tartar and Gailey [1922] who found that in the case of mastic sol precipitation by acid always occurred at a p_H of about 2·6, independently of the actual acid used, or the actual amount added. At any p_H greater than 2·6, no precipitation occurred. We have obtained exactly similar results with a 'benzoin' sol, so that a protective effect of the acid anion would not appear to occur. Tartar and Gailey have also shown, using the same mastic sol, that in precipitation by salts, the p_H of the salt solution is of great importance and may largely account for differences attributed to a special protective effect of the anion. With, for example, sodium acetate, the p_H is high, and high concentrations of the salt are required to precipitate, but if the p_H is brought to a constant value by adding the acid corresponding to the salt, *e.g.* acetic acid in the case of sodium acetate, the same equivalent amount of salt is effective. We have treated the question from a slightly different point of view, as we have found the amounts of a standard electrolyte, sodium chloride, required to precipitate at various hydrogen ion concentrations, and compared the amounts of other salts necessary to precipitate with the amount of sodium chloride required at the p_H produced by the new salt. Our results, it will be seen, confirm those of Tartar and Gailey.

Closely connected with the precipitating effect of gelatin on 'benzoin' is its protective power. This protective action of gelatin is of course well known and has been extensively investigated, particularly with regard to the gold sol. From our observation on the effect of gelatin on 'benzoin' it will be seen that considerably higher concentrations of gelatin are required to protect than to precipitate, confirming the statement made by Neisser and Friedemann.

The obvious suggestion is that with increasing concentrations of gelatin, increasing amounts are absorbed on the 'benzoin' particles. If the gelatin is positively charged, the negative charge on the 'benzoin' particles should decrease, until the zone of precipitation occurs. With more gelatin, the particles now coated with gelatin ought to be positively charged and they should commence to show protection. Full protection should set in when sufficient gelatin is absorbed to form a film round the particles, that is, at higher concentration than is sufficient to cause precipitation. If, however, the gelatin is negatively

charged, no such zone of precipitation ought to occur. According, however, to the work of Loeb [1922, 1] gelatin is positively charged on the acid side of the isoelectric point ($p_H = 4{\cdot}7$), and negatively charged on the alkaline side of the isoelectric point. Hence, on the alkaline side of the isoelectric point, no precipitation ought to occur. It was, therefore, of the greatest interest to investigate the effect of change of the p_H on the precipitating and protecting effect of gelatin. The details will be found in the experimental part of the paper, but it may be said that, in general, the results to be expected are obtained. In particular, the zone of precipitation of 'benzoin' by gelatin suddenly disappears when the p_H exceeds 4·7. On the other hand, if oxyhaemoglobin, which behaves similarly to gelatin in precipitating 'benzoin,' is used, the zone persists until the p_H is over 6.

The experimental work which follows naturally divides itself into four parts:

1. General.
2. Precipitation of the sol by electrolytes.
3. Precipitation of the sol by gelatin.
4. Protection of the sol by gelatin.

I. General.

The 'benzoin' sol used by Gullain, Laroche, and Lechelle [1922], is prepared from an alcoholic extract of Sumatra gum benzoin, 10 g. of powdered crude Sumatra gum benzoin and 100 cc. of absolute ethyl alcohol, being allowed to stand at ordinary temperature for forty-eight hours. 0·3 Cc. of the filtered extract, which can be kept indefinitely, is added drop by drop with vigorous stirring to 20 cc. of distilled water at 35°. The white milky sol obtained is cooled to room temperature when it is ready for use. As, however, it was not homogeneous even to the naked eye but possessed a scum of fine particles, slight modifications were introduced in order to obtain a uniform sol for experimental purposes. After a few experiments it was found that the sol, if prepared at 55° instead of 35°, could be filtered readily through a folded No. 1 Whatman filter paper, whereas the sol prepared at 35° did not filter readily and yielded a very weak filtrate. Accordingly, the sol used in the following experiments was prepared by adding, at 55°, 0·3 cc. of the alcoholic extract drop by drop to 20 cc. of water, with vigorous stirring, care being taken to drop the extract directly into the liquid without touching the side of the vessel. The fluid obtained was filtered at once through a folded filter paper, and the filtrate used after cooling to room temperature. In the initial stages of the work, fresh sol was prepared for each experiment, but later it was found that this was not necessary as the properties of the sol did not change on standing for a few days.

The sol so prepared is slightly less sensitive to precipitation by sodium chloride than is the original, as shown by the following table. 1 Cc. of sol is

added to each of a series of small test-tubes, each containing 1 cc. of a solution of sodium chloride. The tubes are immediately shaken and allowed to stand overnight at room temperature. The strengths of the solutions of sodium chloride in the various tubes are given.

Table I.

Effect of mode of preparation on sensitiveness of sol to salt precipitation.

Concentration of NaCl	0·01 %	0·02 %	0·03 %	0·04 %	0·05 %	0·06 %
Sol prepared at 35°, unfiltered	0	2	4	4	4	4
,, 35°, filtered	0	0	0	0	2	4
,, 55°, unfiltered	0	0	2	3	4	4
,, 55°, filtered	0	0	0	0	4	4

The degree of precipitation in the tubes in these and in the following experiments is denoted by numbers:

4 = complete precipitation;

3 = almost complete precipitation, a slightly opalescent fluid being left;

2 = partial precipitation, the fluid being milky;

1 = slight precipitation, some of the precipitated sol being visible at the foot of the tube, but little change being observable in the liquid left. Sometimes differences in opacity were observed, without definite flocculation or sedimentation, but usually these have been neglected.

The test-tubes were of hard glass, and before being used in any experiment were thoroughly cleaned by standing at least sixteen hours in sulphuric acid-dichromate mixture, followed by washing out each one separately about twelve times in tap-water, after which they were immersed for some time in tap-water and then in freshly distilled water. They were touched throughout only with clean nickel tongs. They were then allowed to drain in crates lined with filter paper.

Unless otherwise stated, the tubes were well shaken after the various solutions had been added, and were then allowed to stand overnight, so that they were read the following day.

The sol, as prepared for these experiments, is an apparently homogenous, milky fluid, of great stability. No apparent settling takes place even after standing for weeks. The sol is definitely acid, 0·3 cc. of $N/50$ NaOH being required to neutralise 10 cc. of it (using methyl red as indicator). When seen under the ultra-microscope, the 'benzoin' particles appear of fairly uniform size, in vigorous Brownian movement, and the observation of Macheboeuf [1921] that they are negatively charged has been verified repeatedly. A very rough count indicated that the diameters of the particles were of the order $0{\cdot}3\mu$; precise estimations were not attempted. They may be seen with the ordinary dark ground illumination apparatus.

The determination of hydrogen ion concentration in the following experiments was carried out colorimetrically using the Clark-Lubs sulphonephthalein series of indicators, an electrometric apparatus not being available [Clark,

1920]. This meant that determinations could be made only in the supernatant fluid, after precipitation had occurred, as it did not appear possible to make readings in the presence of the sol. It is unlikely, however, that the hydrogen ion concentration of the supernatant fluid differs from that of the mixture before precipitation, as it is shown later that in the case of precipitation by calcium chloride or by silver nitrate, the amount of calcium or silver carried down with the precipitate is at most very small. Now a change of 0·1 in the p_H means a change of about 25 % in the actual hydrogen ion concentration, and so it is very improbable that hydrogen ions are absorbed by the precipitate in quantities sufficient to cause any significant change in the p_H.

Readings were at first made with buffer mixtures, but it was found that very good readings were obtained by the use of the coloured plate given in the book by Clark [1920]. With practice good and consistent readings are obtained, and most of the readings recorded have been obtained by this method.

II. Precipitation of the Sol by Electrolytes.

Acids. Tartar and Gailey [1922] found that the precipitation of mastic sol by acids was largely if not entirely, dependent on hydrogen ion concentration, precipitation occurring when the p_H of the solution was less than 2·6 or 2·8. The following experiment shows that 'benzoin' gives a similar result.

To 5 cc. of acids of various strengths was added 5 cc. of 'benzoin' sol. After precipitation was complete the p_H was determined in the supernatant fluid of the tube which showed complete precipitation in the presence of the smallest amount of acid.

The results obtained were as follows:

Hydrochloric acid		2·6
Acetic	,,	2·8
Sulphuric	,,	2·6
Oxalic	,,	2·8
Citric	,,	2·8

It will be seen that, within the experimental error of the methods employed, precipitation by acids depends on the hydrogen ion. The mineral acids appear to be slightly less effective than the organic acids, but it is very doubtful if the difference has any significance. In any case, it is usually the ions of the organic acids that are said to exert antagonistic and protective action [cf. Taylor, 1920], as much larger amounts of equivalent solutions of the sodium salts of these acids are required to precipitate than of sodium chloride. They certainly seem to exert no protective action against precipitation by the hydrogen ion. It would, in fact, appear that organic anions as such do not protect, but that, as Tartar and Gailey suggest, the anomalous behaviour of their salts is due to alkalinity.

To investigate this further a large number of experiments has been carried out. Determinations have been made of the minimum amounts of various salts which, in a volume of 1 cc., were required to produce precipitation of 1 cc.

of 'benzoin' sol. The maximum amount failing to produce precipitation is also recorded. In Table II are recorded the percentage strength of the solutions added and also the amount of salt in these solutions calculated in terms of gram equivalents $\times 10^6$ (b and b_1 in Table II).

Table II.

Precipitation by electrolytes.

	Maximum salt concentration not giving precipitation		Minimum salt concentration giving precipitation		
	a = actual concentration of salt solution %	b g. Eq. $\times 10^6$ per cc.	a_1 = actual concentration of salt solution %	b_1 g. Eq. $\times 10^6$ per cc.	p_H of supernatant fluid
NaCNS	0·025	3·1	0·05	6·2	4·2
NaBr	0·05	4·9	0·075	7·3	4·2
$Na_2Fe(CN)_5NO$. $2H_2O$	0·075	5·0	0·125	8·4	4·2
Na_2SO_4 . $10H_2O$	0·14	8·7	0·15	9·3	—
NaI . $2H_2O$	0·125	6·7	0·175	9·4	4·2
$NaIO_3$	0·15	7·6	0·2	10·1	4·2
NaCl	0·04	6·8	0·06	10·2	4·2
$NaClO_3$	0·075	7·0	0·125	11·7	4·2
$NaNO_3$	0·05	5·9	0·1	11·8	4·2
$NaClO_4$	0·125	10·2	0·2	11·3	4·2
$Na_4Fe(CN)_6$. $12H_2O$	0·15	11·5	0·225	17·3	5·4
NaF	0·05	11·9	0·875	20·8	4·5
$Na_2C_2O_4$	0·2	29·8	0·25	37·3	5·4
Na_2 tartrate . $2H_2O$	0·3	26·3	0·5	43·8	5·4
$NaNO_2$	0·15	21·7	0·3	43·5	5·6
Na acetate . $3H_2O$	1·0	73·5	2·0	147·1	6·8
Na_3 citrate . $5\frac{1}{2}H_2O$	1·5	126·1	2·0	168·1	7·4
NaCN	No precipitation up to 5 % NaCN				
$K_2Cr_2O_7$	0·005	0·34	0·015	1·0	3·8
$K_3Fe(CN)_6$	0·0625	5·7	0·075	6·8	4·0
KIO_3	0·1	4·7	0·15	7·0	4·2
KBr	0·0875	7·3	0·1	8·4	4·2
K_2SO_4	0·05	5·7	0·075	8·6	4·2
KI	0·1	6·0	0·15	9·0	4·0
KCl	0·06	8·0	0·07	9·4	4·2
KNO_3	0·05	4·9	0·1	9·8	4·0
$KClO_3$	0·1	8·2	0·125	10·2	4·2
KCNS	0·075	7·7	0·125	12·9	4·2
$K_4Fe(CN)_6$. $3H_2O$	0·15	14·2	0·2	19·0	5·2
KF . $2H_2O$	0·14	14·8	0·18	19·1	4·3
K_2 oxalate . H_2O	0·2	24·1	0·3	36·1	5·2
K_3 citrate . H_2O	1·0	92·5	2·0	185·0	7·2

It will be seen that the figures obtained for salts with univalent cations were not constant. This is in agreement with the experimental results of others. Measurements were made of the p_H of the supernatant fluid in the tubes which just showed complete precipitation. It was then found that there was a marked relationship between the p_H of the solution and the amount of the salt required to produce precipitation of the solution.

This agrees well with the results of Tartar and Gailey [1922] for mastic sol. They added equivalent amounts of various salts to the sol and then determined the p_H to which the mixtures required to be brought in order to cause precipitation. It was found to be the same in all cases. In view of this it became of interest actually to determine the effect of p_H on precipitations

of 'benzoin' by sodium chloride. Solutions of alkali and acid of various strengths were prepared and 0·5 cc. of these added to each of a row of tubes. To each tube in the various rows were then added 0·5 cc. of sodium chloride solution of increasing strength and then 1 cc. of 'benzoin.' After precipitation was complete, the p_H of the supernatant fluid was measured in the tube showing complete precipitation with the smallest amount of sodium chloride in each row (see Table III). The amount of sodium chloride present was

Table III.

Effect of p_H on precipitation by NaCl.

		Concentration of NaCl											
		4 %	2 %	1 %	0·5 %	0·25 %	0·125 %	0·06 %	0·03 %	0·015 %	0·007 %	0·0037 %	0·0019 %
Concentration of hydrochloric acid	*N*/400	—	—	—	—	4	4	4	4 (p_H 2·8)	4	4	4	4
	N/800	—	—	—	—	4	4	4	4 (p_H 3·2)	3	2	0	0
	N/1600	—	—	—	—	4	4	4 (p_H 3·4)	4	2	0	0	0
	N/3200	—	—	—	—	4	4	4 (p_H 3·6)	2	0	0	0	0
	N/12800	—	—	—	—	4	4	4 (p_H 3·8)	0	0	0	0	0
	Distilled water	—	—	—	—	4	4	4 (p_H 4·1)	0	0	0	0	0
Concentration of sodium hydroxide solutions	*N*/12800	—	—	—	—	4	4	4 (p_H 4·4)	0	0	0	0	0
	N/6400	4	4	4	4	4	4 (p_H 4·4)	1	0	0	0	0	0
	N/3200	4	4	4	4	4	4 (p_H 4·6)	0	0	0	0	0	0
	N/2500	4	4	4	4	4	4 (p_H 4·6)	0	0	0	0	0	0
	N/2000	4	4	4	4	4 (p_H 5)	0	0	0	0	0	0	0
	N/1600	4	4	4	4	4 (p_H 5)	0	0	0	0	0	0	0
	N/1400	4	4	4 (p_H 6·3)	2	0	0	0	0	0	0	0	0
	N/1200	4	4	4 (p_H 6·4)	0	0	0	0	0	0	0	0	0
	N/800	4	4	3 (p_H 6·8)	0	0	0	0	0	0	0	0	0
	N/600	4	4 (p_H 7·2)	0	0	0	0	0	0	0	0	0	0
	N/400	4 (p_H 7·4)	3	0	0	0	0	0	0	0	0	0	0
	N/200	4 (p_H > 9·5)	0	0	0	0	0	0	0	0	0	0	0

calculated. It was found that the amount of sodium chloride required to produce precipitation increased with the increase in p_H. It is, therefore, necessary in order to compare the efficiency of salts as precipitants to do so at the same p_H. In Fig. 1 curves have been drawn representing the amount of sodium chloride required at various p_H. With this is recorded a curve in which the amount of different sodium salts required to produce precipitation is plotted against the p_H of the supernatant fluid in the tube where complete precipitation just occurs.

It will be seen that though the correspondence of the curves is not quite exact, the p_H may at least account for most of the discrepancies between the various salts.

In the case of sodium citrate (p_H 7·4) the figure found is 192, whereas at that p_H the amount of sodium chloride required is 250. The difference is probably not more than the experimental error, which is large because of the small degree of buffering of the sodium chloride solutions. Moreover, the colorimetric estimations are only approximate.

One or two interesting points which arise from our experiments may be commented on here.

With ferric chloride difficulties in estimating the minimum amount of salt required to precipitate were encountered, because two zones of precipitation occurred. In the zone at higher concentrations (0·1 % solution) the p_H is 2·8, a degree of acidity which could of itself produce precipitation. In the second

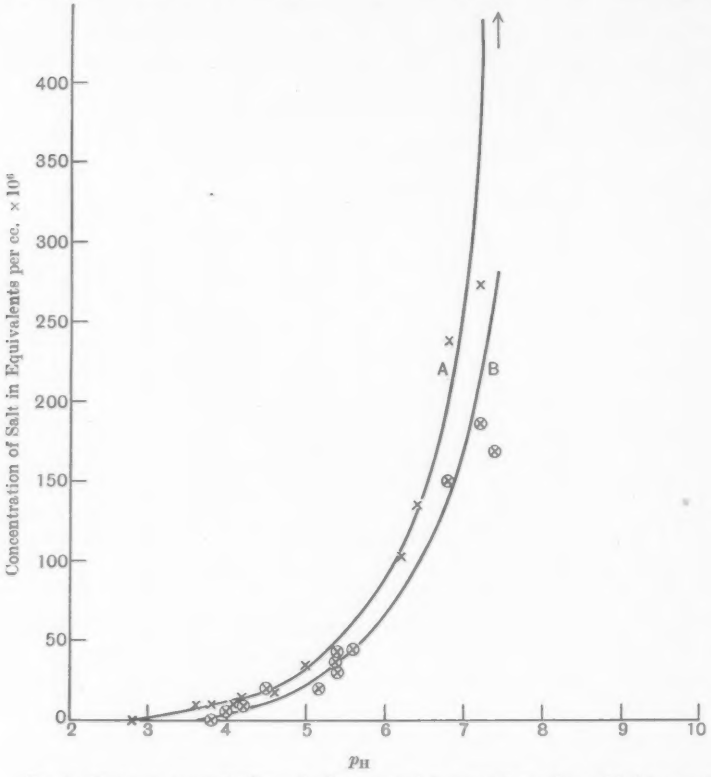

Fig. 1. Minimum concentrations of salts required to precipitate when added to equal volume of 'benzoin' sol.

×. Concentrations of sodium chloride, the p_H being adjusted by addition of acid or alkali.

⊗. Concentrations of various salts as detailed in Table II. p_H is that of supernatant fluid after precipitation of 'benzoin.'

Curve *A* represents concentrations of sodium chloride just sufficient to cause precipitation at various p_H.

Curve *B* is drawn from results obtained with various salts other than sodium chloride.

zone of precipitation (at 0·0015 %) the precipitation is possibly due to colloidal ferric hydroxide as in the intermediate region no precipitation occurs and the 'benzoin' particles have a positive charge. Similar results were obtained by Neisser and Friedemann [1904] with 'mastic' sol.

With aluminium sulphate, however, between concentrations of 10 % and 0·00007 % no such second zone appeared as in the case of ferric chloride.

Indeed aluminium sulphate precipitates the sol at all concentrations greater than that at which it first produces precipitation, and causes no reversal of sign of charge on the particles. Such reversal of sign in the case of ferric chloride is possibly due to adsorption of colloidal iron hydroxide on the particles. At the low concentration used, most of the ferric chloride will be hydrolysed, and so will probably be present as ferric hydroxide sol. In that case the phenomena observed will be similar in nature to the precipitation observed with gelatin, and it is being investigated at greater length.

It is to be noted that no precipitation was obtained with mercuric chloride in concentrations up to 10 %, presumably because of the low degree of ionisation of this salt. Similar results have been previously obtained using other sols, and this very strongly indicates the ionic nature of the precipitation.

With sodium cyanide and potassium cyanide no precipitation was obtained. This is no doubt due to the strong alkalinity of the solutions.

Experiments were carried out with silver nitrate and also calcium chloride solutions as precipitants of 'benzoin' in order to determine any change in the salt concentration in the supernatant fluid after precipitation of the sol.

Calcium chloride solution (approx. 0·06 %) was added to an equal quantity of the sol. After standing overnight precipitation of the sol was complete. The clear supernatant fluid was pipetted off. The amount of calcium present was estimated by the usual method—precipitation as oxalate and titration of the oxalic acid with potassium permanganate solution. In 150 cc. of the supernatant fluid was found 0·0131 g. of calcium. An estimation of the calcium present in 150 cc. original solution gave 0·0259 g. Ca, or, diluted one in two, 0·0130 g. So that, within the limits of experimental error the determinations were identical.

Similar results were obtained when silver nitrate was used as the precipitating salt, the silver in this case being estimated volumetrically.

The amount of the precipitating ion removed during the process of precipitation was apparently too small to be detected by the methods. As noted above (p. 639), these results give a basis for the practice of measuring the p_H of the supernatant fluid as the p_H at which precipitation actually takes place.

Effect of Concentration of Sol on Precipitation by Electrolytes.

Burton and Bishop [1920] and Burton and McInnes [1921] have formulated the following laws:

(1) In the case of univalent ions, the amount required to cause precipitation increases as the solution is diluted.

(2) In the case of bivalent ions, no such increase is observed.

(3) In the case of tervalent ions, the amount required is approximately proportional to the concentration of the solution.

Weiser and Nicholas [1921] have, however, cast doubt on the general validity of these laws, a doubt which is apparently shared by Kruyt and van der Spek [1919]. We have determined the minimum concentrations of sodium

chloride, copper sulphate and aluminium sulphate solutions required to produce precipitation when added to 'benzoin' sols of the following strengths:

(1) 'Benzoin' of double[1] the usual strength (*i.e.* 3 cc. of alcoholic extract added to 100 cc. of water).

(2) 'Benzoin' of double the usual strength, diluted 1 in 2 with distilled water.

(3) 'Benzoin' of double the usual strength, diluted 1 in 20 with distilled water.

The results are given in Table IV, and the amount of sodium chloride required in the case of 'benzoin' of the usual strength is given for comparison.

Table IV.

Effect of concentration of sol on precipitation by kations of different valency.

Nature of sol	Concentration of solution which produces precipitation when added to an equal volume of sol		
	NaCl %	$CuSO_4$ %	$Al_2(SO_4)_3$ %
'Benzoin,' ordinary strength	0·05–0·06	—	—
" double "	0·04–0·05	0·03–0·05	0·007–0·015
" " " diluted 1/2	0·05–0·08	0·04–0·06	0·007–0·015
" " " " 1/20	0·1 –0·2	0·08–0·1	0·015–0·03

It would appear that these laws are not here applicable.

In all cases, an increased concentration of salt is required to precipitate diluted solutions.

Dilute sols are also apparently slightly less sensitive to precipitation by hydrogen ions. Direct experiment showed a slight but constant difference between the p_H required to precipitate a concentrated solution and that effective for a dilute one. Whereas a double strength 'benzoin' sol is precipitated when a p_H of 2·8 is reached, the same sol diluted 1 in 20 requires a p_H of 2·6 to precipitate it. This shows that the greater sensitiveness of the stronger solution to salts is not due altogether to its higher p_H, although there is actually a slight difference in the p_H of the concentrated and dilute solutions. The following experiment in which the salt content is kept constant and the p_H varies, also shows that this difference in p_H is not sufficient to account for the difference in sensitiveness to electrolytes. 1 Cc. of a dilute sol (double strength diluted 1 in 10) was added to each of ten tubes, each containing 1 cc. of sodium chloride of the concentration (0·06 %) required to produce precipitation in an ordinary sol at a p_H of 4·2. The sodium chloride solution contained acetic acid of strengths 1 %, 0·5 %, 0·25 %, etc., in the various tubes so as to vary the p_H. Precipitation appeared in those tubes having a p_H of 3·6 or less and not in the others. The dilute sol, therefore, appears definitely less sensitive to electrolyte precipitation than the normal one, which is precipitated by an equal volume of 0·06 % sodium chloride at a p_H 4·2.

[1] This 'benzoin' sol double the usual strength was used so that, when diluted 1 in 20, the resulting liquid was still opaque enough to show precipitation readily when that had occurred,

III. Precipitation of 'Benzoin' by Gelatin and Oxyhaemoglobin.

As mentioned in the introduction it has been found that gelatin at certain dilutions produces precipitation of 'benzoin' sols. A 1 % solution of iso-electric gelatin was prepared by the method described by Loeb [1922, 1]. On ignition this was found to leave an ash weighing 0·08 % of the dry gelatin. A series of dilutions of gelatin in distilled water was prepared varying from 1/10,000, 1/20,000 to 1/5,120,000. Each tube contained 1 cc. of gelatin solution and to this was added 1 cc. of 'benzoin' sol. Precipitation occurred in the tubes containing gelatin solution 1/320,000 and 1/640,000 and in the tubes containing gelatin solutions above and below these concentrations no precipitation occurred.

A series of experiments was carried out to study the effect of variation of p_H on the zone of precipitation. For the purpose a series of dilutions of gelatin, hydrochloric acid and sodium hydroxide were prepared. To each of a series of tubes were added 0·5 cc. of gelatin solution of various strengths, 0·5 cc. of the appropriate acid or alkali solution and finally 1 cc. of 'benzoin.' Readings were made when precipitation was complete and the p_H was estimated in the supernatant fluid. The figures in Table V represent the concentrations of acid or alkali and of gelatin in the mixture before addition of the 'benzoin.' The concentrations originally prepared were double that recorded.

Table V.

Precipitation of 'benzoin' by gelatin.

	Concentration of gelatin	1/2500	1/5000	1/10,000	1/20,000	1/40,000	1/80,000	1/160,000	1/320,000	1/640,000	1/1,280,000	1/2,560,000	1/5,120,000	No gelatin
Concentration of sodium hydroxide	*N*/800	0	0	0	0	0	0	0	—	—	—	—	—	—
	N/1600	0	0	0	0 (−)	0 (−)	0	0	—	— (−)	—	—	—	—
	N/3200	0	0 (+)	0	0 (+)	1 (?+)	4	4 (p_H 4·7)	4	1	0	0	—	0
	N/12800	0	0 (+)	0	0 (+)	0 (+)	0 (+)	1	4 (p_H 4·1)	4 (p_H 4·1)	0	0 (−)	—	0
	N/51200	0	0	0	0	0	0	0	3	4 (p_H 4·1)	0	0	—	0
	H_2O	0	0	0	0 (++)	0	0	0 (+)	2	4 (p_H 4·1)	0	0	—	0 (−)
Concentration of hydrochloric acid	*N*/51200	0	0	0	0	0	0	0	1	4 (p_H 4·1)	1	0	—	0
	N/12800	—	—	—	—	0	0	0	1	4 (p_H 3·8)	1	0	—	0
	N/3200	—	—	—	0 (+++)	0	0 (++)	0	1	4	4 (p_H 3·6)	2	0	0
	N/1600	—	—	—	—	—	—	0	1	4	4 (p_H 3·5)	3	0	0
	N/800	—	—	—	—	—	—	0	1	4	4 (p_H 3·2)	4	3	0
	N/400	—	—	—	—	—	—	0	1	4	4 (p_H 2·8)	4	4	4
	N/200	—	—	—	—	—	0	0	0	1	—	—	—	—
	N/100	—	—	—	—	—	0	0	1	2	—	—	—	—
	N/40	—	—	—	—	—	0	1	3	4	—	—	—	—
	N/10	—	—	—	0	0	1	4	4	4	—	—	—	—

(+) and (−) indicate sign of charge on the particles.

It may be noted that in these experiments as in those on precipitation by electrolytes, the p_H recorded is that of the supernatant fluid. The 'benzoin' solution is itself acid, and so the p_H of the gelatin solution before the 'benzoin' is added is of no value in giving the p_H at which the precipitation actually occurs. The p_H of the supernatant fluid suggests itself as the best measurement of the p_H at which precipitation occurs.

Fig. 2 shows the p_H plotted against concentration of gelatin. It will be seen that as the acidity is increased a decrease occurs in the concentration of gelatin at which precipitation takes place until a p_H of 2·8 is reached when the zone

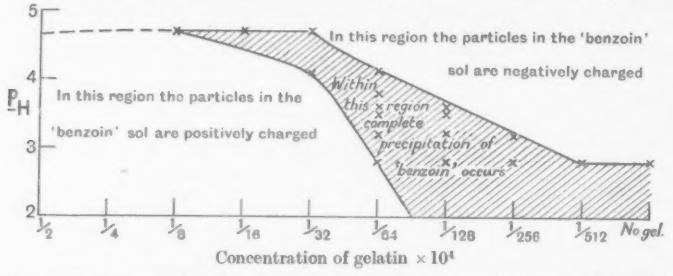

Fig 2. Illustrating effect of p_H on precipitation of 'benzoin' by gelatin. (N.B. The concentration of gelatin given is that of solutions before addition of equal volume of 'benzoin' sol.)

effect disappears, the acid itself precipitating the 'benzoin' solution, the gelatin now appearing in the rôle of a protective colloid. On increasing the p_H on the other hand the precipitating zone is found in higher concentrations of gelatin. As the isoelectric point of gelatin is reached (p_H 4·7) the zone broadens, but when this is passed no further precipitation of the 'benzoin' is observed.

Precipitation of 'Benzoin' by Oxyhaemoglobin.

In view of the interest attached to the precipitation of 'benzoin' by gelatin, more particularly with regard to the importance of the isoelectric point, and its predominance in the effect, it appeared to be very desirable to investigate in a similar way, a protein having a markedly different isoelectric point. Serum globulin and serum albumin were both investigated; precipitation took place at a p_H up to the isoelectric point, and beyond this, there was no precipitation. This, however, will be discussed at greater length in the succeeding paper. Oxyhaemoglobin is of greater interest as its isoelectric point differs from that of gelatin and is given as 6·6, and, therefore, these zones of precipitation should not exist at p_H exceeding 6·6, but should exist up to about that point.

A solution of oxyhaemoglobin was prepared by washing well with 0·8 % sodium chloride solution, red blood cells obtained from citrated sheep's blood, so that the washings finally gave no test for protein. The cells, which weighed moist 8·01 g., were added to distilled water, and the resulting oxyhaemoglobin solution dialysed for over thirty-six hours in a collodion sac against running tap-water. The final volume of oxyhaemoglobin solution was 85 cc. This is of course not quite pure oxyhaemoglobin solution but, particularly in high dilutions, the amount of impurity present is probably very small.

An experiment exactly similar to the experiment with gelatin described on p. 645 was carried out with this solution. The results are given in Table VI. It may be remarked that variations in different experiments were

occasionally noticed in the tubes in the first four rows. This is probably to be attributed to the great sensitiveness at this point to the slight change in reaction.

Table VI.

Precipitation by Oxyhaemoglobin.

Concentration of oxyhaemoglobin	1/6·25	1/12·5	1/25	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200	1/102400	1/204800	1/409600
Concentration of sodium hydroxide N/200	—	—	—	—	—	0	0	0	0	0	0	0	0	0	0	0	0
N/400	—	—	—	—	—	0	0	0	0	0	0	0	0	0	0	0	0
N/800	4	2	0	4 (p_H 6·3)	0	0	0	0	0	0	0	0	0	0	0	0	0
N/1000	4	4	2	4 (p_H 6·4)	2	0	0	4 (p_H 6·1)	0	0	0	0	—	—	—	—	—
N/1200	4	4	4	4	4 (p_H 6·2)	2	2	4 (p_H 6·4)	0	0	0	0	—	—	—	—	—
N/1400	4	4	4	4	0	0	0	4 (p_H 5·7)	4 (p_H 5·6)	0	0	0	—	—	—	—	—
N/1600	4	4	4 (p_H 5·8)	0	0	0 (+)	0	4	4 (p_H 5·8)	0	0	0	0	—	—	—	—
N/3200	—	—	—	—	—	0	0	0	0	4 (p_H 4·8)	4	0	0	—	—	—	—
N/6400	4	1	0	0	0	0	0	0	0	0	4 (p_H 4·8)	2	—	—	—	—	—
N/12800	—	—	—	—	—	—	—	0	0	0	4 (p_H 4·4)	0	0	0	0	—	—
Distilled water	—	—	—	—	—	—	—	0	0 (+)	0	4 (p_H 4·2)	4	0	0	0 (−)	—	—
Concentration of hydrochloric acid N/3200	—	—	—	—	—	—	—	—	0	0	0	4 (p_H 3·8)	4	0	0	0	—
N/1600	—	—	—	—	—	—	—	—	—	0	0	4 (p_H 3·4)	4	0	0	0	—
N/800	—	—	—	—	—	—	—	—	—	—	0	4 (p_H 3)	4	4	3	0	0
N/400	—	—	—	—	—	—	—	—	—	—	0	2	4 (p_H 2·8)	4	4	4	4

Fig. 3 shows p_H plotted against concentration of oxyhaemoglobin.

It will be seen that the zone of precipitation which at a p_H of 3·8 appears in the tube containing the oxyhaemoglobin solution diluted 1/12,800 gradually moves to the left with increasing p_H until the p_H rises to about 6–6·4, when

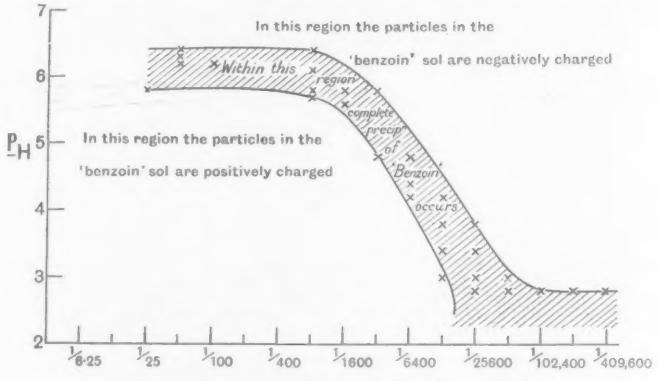

Fig. 3. Illustrating effect of p_H on precipitation of 'benzoin' by oxyhaemoglobin. (N.B. The concentration of oxyhaemoglobin is given in terms of an original solution prepared as described in the text, and is the concentration before addition of an equal volume of 'benzoin' sol.)

precipitation occurs at 1/800. A zone of precipitation at this p_H occurs with all concentrations of haemoglobin solution down to 1/6·25. The absence of any precipitation beyond the isoelectric point and presence of it up to the isoelectric point is very marked.

The effect of alteration in the reaction of the gelatin or oxyhaemoglobin in precipitation of 'benzoin' is a peculiar one. There is a general change in the position of the zone of precipitation with change of p_H. This is readily understood if it be assumed that the primary effect of change of the p_H is on the protein. In accordance with the work of Loeb gelatin exists on the acid side of the isoelectric point as a positively charged kation, on the alkaline side as an anion. If the effective precipitating agent is the positively charged gelatin ion it can be understood that little or no precipitation will occur on the alkaline side of the isoelectric point. The alteration in the position of the zone of precipitation is probably due to the fact that an optimum concentration of the gelatin is required for precipitation. This amount will be found at lower concentrations as the p_H decreases, as a greater part of the gelatin exists with a positive charge at the higher acidity.

That there may be some effect on the sol must be granted as acid in excess causes its precipitation, while excess of alkali dissolves it. But that this effect is not the primary one is shown by an experiment in which varying amounts of sodium chloride solution are added to the gelatin, instead of acid.

Table VII shows the result of such an experiment.

Table VII.

Influence of sodium chloride on the precipitation of 'Benzoin' by Gelatin.

The concentrations given are those of the sodium chloride and gelatin after mixing and before adding the 'benzoin.'

Concentration of sodium chloride	Concentration of gelatin												
	1/2000	1/4000	1/8000	1/16,000	1/32,000	1/64,000	1/128,000	1/256,000	1/512,000	1/1,024,000	1/2,048,000	1/4,096,000	Water
1·0 %	0	0	2	3	4	4	—	—	—	—	—	—	—
0·5 %	0	0	1	2	4	4	—	—	—	—	—	—	—
0·1 %	0	0	0	1	4	4	4	—	—	4	4	4	4
0·04 %	0	0	0	0	1	4	4	4	4	4	3	0	0
0·02 %	0	0	0	0	0	4	4	4	4	3	0	0	0
0·005 %	0	0	0	0	0	0	3	4	4	3	0	0	0
0	0	0	0	0	0	0	0	4	4	0	0	0	0

It will be seen that the effect of sodium chloride on the zone of precipitation of 'benzoin' by gelatin is merely to widen it. Sodium chloride at such concentrations presumably has little effect on gelatin. It has at least no such powerful influence in altering its properties as has acid or alkali. On the other hand, the effects of sodium chloride and of hydrochloric acid on the 'benzoin' solution in the absence of gelatin are qualitatively the same, they either precipitate it or increase its sensitiveness. Hence there seems to be little doubt that the action of the acid in the above experiment must be primarily on the gelatin, because, if it were on the 'benzoin,' we should expect an effect, similar to that of the salt. This of course is also borne out by the determining influence of the isoelectric point of the protein.

Effect of Concentration of the Sol on Precipitation by Gelatin.

Neisser and Friedemann [1904] and Biltz [1904] showed that a quantitative relationship held in the mutual precipitation of oppositely charged colloids, and that this is related to a neutralisation of the charge on the particles or to a reduction of the potential differences between the disperse phase and the continuous phase. If the precipitation of 'benzoin' (negatively charged) by gelatin (positively charged) is of this nature, change in the concentration of 'benzoin' ought to cause a similar change in the concentration of gelatin at which precipitation occurs. That this actually occurs is shown by an experiment, the results of which are given in Table VIII.

Table VIII.

Effect of concentration of sol on precipitation by Gelatin.

1 cc. of the gelatin solution was added to 1 cc. of 'benzoin' solution, the mixture shaken and allowed to stand overnight.

Concentration of gelatin solution $\times 10^4$	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
'Benzoin,' ordinary strength	0	0	0	0	4 (p_H 4·2)	4	0	—	—	—
" double "	0	0	0	4	4 (p_H 4·2)	2	0	0	—	—
" " " diluted 1/2	0	0	0	0	4 (p_H 4·2)	4	0	0	—	—
" " " " 1/4	0	0	0	0	4	4 (p_H 4·4)	0	0	0	—
" " " " 1/20	0	0	0	0	0	2	4 (p_H 4·6)	0	0	0

It will be seen that a sol in a dilution of 1/20 of double strength (= 1/10 dilution of ordinary strength) requires gelatin 1/1,280,000 to produce maximum precipitation (p_H 4·6). Referring to Table V it will be seen that the maximum precipitation of a sol of ordinary strength with this p_H occurs in a concentration of gelatin of 1/160,000. Approximately, therefore, diluting the solution ten times reduces the optimum amount of gelatin to one-eighth of the amount required for the more concentrated solution, or, within the error of the experiment, the same actual amount of gelatin is required to precipitate the same actual amount of 'benzoin' at different dilutions.

Charge on the 'Benzoin' Particles.

For the purpose of observing the nature of the charge on the particles a Siedentopf-Zsigmondy slit ultramicroscope was employed. The ordinary cell was used and two fine platinum wires were introduced into this, one from each end in such a way that the end of each wire was about 2 inches from the part of the fluid observed. It was found that, to produce a definite movement, the 220-volt current was convenient, a switch, change over key and lamp being included in the circuit. The circuit was closed and the movements of the particles in the field observed immediately. The current was then reversed and a second observation made. It is essential that observations should be made immediately after closing the circuit so that interference by electrolyte disturbances may be avoided. It was found that this method was particularly useful where, as in the case of experiments with cerebrospinal fluid, only very small quantities (about 1 cc.) of liquid were available. In the crude form described, quantitative estimations were, of course, not possible but for quali-

tative determinations the arrangement was quite satisfactory. The results of such observations are indicated in Tables V and VI.

On the alkaline side of the isoelectric point of gelatin (p_H 4·7) the particles were invariably negatively charged in all concentrations of gelatin observed.

On the acid side of the isoelectric point in those concentrations of gelatin which are too great to cause precipitation, where in fact the 'benzoin' is protected, the charge on the particles is positive. The boundary between positively and negatively charged particles is within the zone of precipitation. For example, in Table V, where gelatin is diluted with distilled water, in dilutions of gelatin up to 1/1,280,000 the particles are negatively charged. At the gelatin dilution of 1/160,000 they have a slight positive charge which becomes more marked as the concentration of gelatin increases. Between these concentrations the zone of precipitation occurs.

An experiment was carried out to determine the effect of tannin in precipitating the mixtures of gelatin and 'benzoin' in the following way. Gelatin solution (1 cc. of 1/40,000 dilution) was added to 1 cc. of various strengths of sodium hydroxide solution. To each tube was added 2 cc. of 'benzoin.' This was allowed to stand overnight and precipitation observed and observation of the charges on the particles were made. The results are given in Table IX.

Table IX.

Effect of Tannin on Benzoin and Gelatin mixtures.

Sodium hydroxide	*N*/200	*N*/400	*N*/800	*N*/1600	*N*/3200	*N*/6400	*N*/12800	*N*/25600	*N*/51200	*N*/102400
Row I	0	0	0	0 (−)	2 (+?)	0 (+)	0	0	0	0

1 cc. of gelatin and 1 cc. of sodium hydroxide solution mixed, and 2 cc. of 'benzoin' added to the mixture. Read as in Row I.

From each tube 1 cc. was taken and an equal volume of a 0·2 % solution of tannin added. The tubes were read after standing overnight as follows in Row II.

Row II	0	0	4 (p_H 4·4)	4	4	4	4	4	4	4

The acidity of the tannin solution has slightly changed the p_H and therefore the electrical charges in the mixtures, but the negative colloid (tannin) is able to precipitate the mixture only at p_H 4·4 and below, under which conditions the particles are positively charged. The 'benzoin' alone is not precipitated by the tannin under the conditions of the experiment, and no visible precipitation is given by gelatin at the concentration employed.

From these experiments it would appear that the 'benzoin' particles combine with the gelatin with the result that their electrical condition is modified. The degree of the modification is dependent on the condition of the gelatin, which again depends on the reaction. Where the charge is neutralised or reduced precipitation occurs. Where, however, the negative charge is reinforced by the gelatin or changed to a positive charge of sufficient magnitude, the sol is protected against precipitation by electrolytes. These results are in substantial agreement with those obtained by Eggerth and Bellows [1922] on bacterial and other suspensions and by Northrop and de Kruif [1922, 1, 2] on bacterial suspensions.

IV. Protection of 'Benzoin' by Gelatin against Precipitation by Electrolytes.

According to the views of Bechhold [1904], and Neisser and Friedemann [1903] the protection of a lyophile colloid by a lyophile depends probably on the formation of a covering of the former round particles of the latter. The result is that the behaviour of the lyophile particles is governed rather by the properties of the protective colloid than by its own. This accounts for the fact, rather striking at first, that colloidal 'benzoin' requires little more gelatin to protect it against a large concentration of salt than is required to protect it against a small quantity. This is shown in the following experiment. Varying solutions of gelatin (1 cc.) 1/100,000, 2/100,000, etc. to 1/10,000 were introduced into a series of test-tubes. To each test-tube was added 0·2 % sodium chloride (1 cc.) and the 'benzoin' (2 cc.). A similar experiment was carried out at the same time with 2 % sodium chloride, and the results are given in Table X.

Table X.

Protection by Gelatin against precipitation by NaCl.

Concentration of gelatin × 100,000		1	2	3	4	5	6	7	8	9	10
Concentration of sodium chloride	0·2 %	4	4	4	0	0	0	0	0	0	0
	2·0	4	4	4	4	0	0	0	0	0	0

It may be noted here that if any such mechanism really does occur, the 'benzoin' particles are completely protected within a few minutes. This is proved by experiments in which to each of a series of ten test-tubes was added 0·5, 1·0, 1·5, 2, etc., up to 5 cc. of gelatin (1/10,000) and distilled water to bring the total volume in each tube to 5 cc. This was followed by the addition to each tube of 5 cc. of 'benzoin' sol, of twice the usual strength (0·6 cc. of alcoholic solution to 20 cc. of water). Six rows, each containing ten tubes, were arranged so that, corresponding to each mixture of gelatin and 'benzoin,' there were six test-tubes, and into each of these six test-tubes was put 1 cc. of the corresponding mixture of 'benzoin' and gelatin, each of the six rows at this stage being of course identical. Ten minutes after the mixture of the 'benzoin' and gelatin, 1 cc. of 2 % sodium chloride was added to each test-tube in the first row and 1 cc. of 0·2 % sodium chloride solution was added to each test-tube in the second row. Thirty minutes after the mixing of 'benzoin' and gelatin, addition was made to the third and fourth row of 1 cc. of salt solution of 2 % and 0·2 % respectively, and after 2½ hours, similar addition to the tubes in the fifth and sixth rows respectively. The results are represented in Table XI, and it will be seen that protection of the 'benzoin' by the gelatin is apparently complete after 10 minutes. It is probable, however, that the protection is not instantaneous, as is shown by the fact that if the salt and gelatin be first mixed and the 'benzoin' then added, slightly different results are obtained, as is also shown in Table XI.

Table XI.

Rate of protection by Gelatin.

Concentration of gelatin × 100,000	1	2	3	4	5	6	7	8	9	10
2 % NaCl added after 10 mins.	4	4	4	4	0	0	0	0	0	0
0·2 % „ „ 10 „	4	4	0	0	0	0	0	0	0	0
2 % „ „ 30 „	4	4	4	4	0	0	0	0	0	0
0·2 % „ „ 30 „	4	4	0	0	0	0	0	0	0	0
2 % „ „ 2½ hours	4	4	4	4	0	0	0	0	0	0
0·2 % „ „ 2½ „	4	4	0	0	0	0	0	0	0	0
'Benzoin' added to mixture of NaCl (0·2 %) and gelatin	4	4	2	0	0	0	0	0	0	0

As is to be expected, there is less protection when the 'benzoin' is added to the mixture of salt and gelatin, as in this case the salt is active as quickly as the gelatin, and effects precipitation before the protective power of the gelatin can fully exert itself.

Experiments were carried out on the amount of gelatin required to protect 'benzoin' of various strengths. A series of five rows each containing ten tubes is arranged. To each tube in each of the first four rows is added 1 cc. of a solution of gelatin, to the first, 1/100,000, to the second, 2/100,000, etc. To the test-tubes of the last row is added 1 cc. of gelatin of strength 1/20,000, 1/40,000, 1/80,000, etc., respectively. Then 1 cc. of 'benzoin' of a certain concentration is added to each tube. Single strength 'benzoin' is added to the tubes of the first row, double strength to the tubes of the second row, double strength diluted 1 in 2, 1 in 4, 1 in 20, to the tubes of the third, fourth and fifth rows respectively. All the tubes are shaken and after 15 minutes 2 cc. of 2 % sodium chloride solution is added to each tube. The results obtained after standing overnight are shown in Table XII.

Table XII.

Effect of concentration of sol on protection by Gelatin.

The concentrations of gelatin given in this table are the concentrations *before* mixing with the 'benzoin.' Sodium chloride (2 %) solution is added to the mixture of 'benzoin' and gelatin after 15 minutes.

Concentration of gelatin × 100,000	1	2	3	4	5	6	7	8	9	10
'Benzoin,' ordinary strength	4	4	2	0	0	0	0	0	0	0
„ double „	4	4	4	4	4	3	2	0	0	0
„ „ „ diluted 1/2	4	4	4	0	0	0	0	0	0	0
„ „ „ „ 1/4	4	0	0	0	0	0	0	0	0	0

Concentration of gelatin × 10,000	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
'Benzoin,' double strength, diluted 1/20	0	0	0	0	0	4	4	4	4	4

It will be noted in the first place that there is not much difference between the single strength 'benzoin' and the double strength diluted 1 in 2. This, of course, is to be expected. On the other hand, the various dilutions of the double strength showed marked differences in the amount of gelatin required to protect them. The double strength 'benzoin' is almost completely protected by 8/100,000 gelatin, giving a final concentration of gelatin in the experiment of $\frac{1}{4}$ (8/100,000) = 1/50,000—the factor $\frac{1}{4}$ being required, since 1 cc. of gelatin is diluted to 4 cc. by the addition of 'benzoin' and of salt, whereas

if the 'benzoin' be diluted 20 times protection occurs at a final concentration of about $\frac{1}{4}$ (1/320,000) = 1/1,280,000, or about 1/25 of the concentration required with 'benzoin' of 20 times the strength of that here used. In fact, the amount of gelatin required to protect is approximately proportional to the concentration of the sol. In interpreting the result it must be remembered that less salt is required to precipitate a concentrated sol than a diluted one, but, on the other hand, if excess of salt be used, as in this experiment, the amount of gelatin required to protect increases very slowly with increase in the actual concentration of the salt (see p. 651). It may be noted, however, that this effect may account for the small deficiency (1/25 instead of 1/20) of gelatin that is required for the precipitation of the dilute sol compared with the concentrated one.

In view of the very great importance of the hydrogen ion concentration on the precipitation effect of gelatin on 'benzoin' it was of interest to investigate its effect on the protective power of gelatin. Solutions of hydrochloric acid, *N*/100, *N*/200, *N*/400...*N*/16,000, *N*/64,000, sodium hydroxide, *N*/200, *N*/400, *N*/800, *N*/1400, *N*/3200, and solutions of gelatin 1/500, 1/1000, 1/1600, 1/3200 were prepared. 0·25 Cc. of acid (or alkali), and the same amount of gelatin, were added to each one of a row of eight tubes, there being eleven rows, one for each strength of acid or alkali and one for water. 0·5 Cc. double strength 'benzoin' was then added to each tube, and readings made of those (marked * on the table) which showed incipient precipitation as indicated by increased opacity. After standing 10 minutes, 1 cc. of sodium chloride solution (2 %) was added to each tube and the tubes were allowed to stand overnight. The results obtained are shown in Table XIII.

Table XIII.

Effect of p_H on protection by Gelatin.

The concentrations given are the final concentrations after mixing 'benzoin,' gelatin and salt solutions.

	Final concentration of gelatin × 1000	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Final concentration of sodium hydroxide	*N*/1600	0	0	0	0	0	4	4 (p_H 6·4)	4
	N/3200	0	0	0	0	0	4	4 (p_H 5·2)	4
	N/6400	0	0	0	0	1	4*	4* (p_H 4·8)	4*
	N/12800	0	0	0	0	4	4	4* (p_H 4·6)	4*
	N/25600	0	0	0	0	2	4	4 (p_H 4·4)	4*
Distilled water		0	0	0	0	2	4	4 (p_H 4·3)	4*
Final concentration of hydrochloric acid	*N*/51200	0	0	0	0	1	4 (p_H 4·2)	4	4
	N/12800	0	0	0	0	0	4 (p_H 4·1)	4	4
	N/3200	0	0	0	0	0	4 (p_H 3·6)	4	4
	N/1600	0	0	0	0	0	4 (p_H 3·3)	4	4
	N/800	0	0	0	0	0	4 (p_H 3)	4	4*

The most interesting result of this experiment is that protection by gelatin is minimum at the isoelectric point. From the point of view that the particles of a protected colloid have many of the properties of the protective substance, this is easily understood. It is interesting to note, however, that Loeb [1923] has obtained similar results in the case of collodion particles coated with gelatin. These show a minimum of stability at the isoelectric point

of gelatin. According to Loeb this is due to the fact that gelatin is least soluble at the isoelectric point, and a similar explanation fits well the results obtained in the above experiment.

One further point is of interest. As shown above (p. 651), rather more gelatin is required to protect against precipitation by 2 % salt solution than against 0·2 % salt solution. The lower part of the table in the above experiment may be looked on as a series of experiments in protection against salt along with a definite amount of acid. It will be seen that up to a point, addition of acid does not increase the amount of gelatin required to protect. In fact, it diminishes it. The only interpretation of this result is that the acid renders the gelatin a more efficient protector, and this may be due to the gelatin anion being a more efficient protector than the uncharged neutral particle. In this case, it is not unreasonable to explain the effect of alkali on the protective power of gelatin as due to a similar increased efficiency of sodium gelatinate compared to unionised gelatin. Of course this comes well into line with the point of view of Loeb, the minimum protection at the isoelectric point being due to the small affinity for water of the isoelectric gelatin particle and the resulting small solubility. At the same time it must be remembered that alkali has itself a protective effect against precipitation by salt (see p. 641), whereas acid tends to precipitate. This may well account for the greater efficiency of gelatin as a protector in presence of alkali compared to acid, as is shown by the fact that, at a p_H of 5·2, 1/64,000 gelatin affords complete protection whereas at a p_H of 4·2 it does not, although the isoelectric point, $p_H = 4·7$, is nearer 5·2 than is 4·2.

Discussion.

Colloidal solutions have for convenience been classified into two types—lyophobe colloids, such as gold sol or 'benzoin' sol which are precipitated by small quantities of salt, and are suggestive of very fine suspensions, and lyophile colloids, on the other hand, such as gelatin solution or a solution of silicic acid which are precipitated only by large quantities of salt, and then redissolved again if the excess of salt is removed. The work of Loeb [1922, 2] has in fact shown that gelatin in certain respects may be regarded as forming a true solution, and that it behaves chemically as an amphoteric substance, combining with acid on one side of the isoelectric point ($p_H = 4·7$) and with bases on the other. The large size of the gelatin aggregate, however, prohibits it from passing through a collodion membrane, and this along with the strong attraction of the gelatin molecules for each other, aiding the formation of micelles, and tending to cause gelatin to swell rather than to dissolve, results in producing the colloidal properties of gelatin.

The 'benzoin' particles, on the other hand, have a negative charge, presumably due to the presence of some organic acid (benzoic acid) on the surface of the resin particles. These acid molecules will naturally ionise to some extent, thus producing a negatively charged particle and a slightly acid solution. The

stability of these particles is largely dependent on the electric charge. Loeb [1922, 2] who uses collodion particles shows that a dispersion of these becomes unstable if the potential difference between particle and liquid is less than about 16 millivolts.

On this basis, the experimental results we have obtained in the investigation of the precipitation of the 'benzoin' sol by gelatin are to be explained. With gradually increasing concentration of positively charged gelatin, there occurs a change in the charge of the 'benzoin' particles, and as the charge is reversed, there occurs the zone of precipitation. It is of great interest to note that the concentration of gelatin at which the zone of precipitation occurs depends essentially on the p_H. On the alkaline side of the isoelectric point, no precipitation occurs at all. The precipitation in fact would clearly seem to depend on the amount of positively charged gelatin present. As the p_H increases and the solution becomes more acid, an increasing proportion of the gelatin present exists as kation, and so the actual concentration at which the gelatin precipitates is decreased.

The work of Walpole [1913] is of interest. Working with an oil emulsion this observer found no precipitation with gelatin alone, but in presence of hydrochloric acid, a zone could be observed, very similar to that described with the 'benzoin' sol without hydrochloric acid. Walpole did not measure the p_H of the solutions, but gives only the normality of the acid employed. Hydrochloric acid, of normality 10^{-2} is alone sufficient to aggregate the emulsion. Between about 10^{-4} and 10^{-2} N precipitation takes place if gelatin be present in concentrations of 10^{-6} to 10^{-7}, the zone tending towards the region of more concentrated gelatin as the concentrations of acid decrease, until at about 10^{-4} N it broadens, and extends from about $10^{-4\cdot2}$ to 10^{-6}. Our own results give a zone of the same general type, and so far confirm the findings of Walpole. In one respect, however, the experiments do not agree. Walpole finds that the line dividing the positively from the negatively charged particles in his diagram, in which the normality[1] of the acid is plotted against the concentration of the gelatin, does not coincide with the zone of precipitation. In our experiments, there was always a different charge on the particles on the two sides of the zone; both in the case of gelatin and of oxyhaemoglobin. Even the case of precipitation by ferric chloride solution (p. 642) which, similarly to gelatin precipitates at high dilutions only over a limited zone, the particles are charged oppositely at the different ends of the zone. In fact, as we have suggested above, this precipitation by ferric chloride probably bears more than a superficial resemblance to the precipitation by gelatin, as the ferric chloride is partially hydrolysed, and the ferric hydroxide exists in a colloidal condition.

Such a reaction between a lyophobe colloid and a lyophile sol is no doubt fairly general for lyophile sols of different kinds. For instance, 'benzoin' is precipitated not only by gelatin, but also by oxyhaemoglobin, serum globulin,

[1] Actually the exponents of normality and concentration respectively.

and serum albumin (see the following paper). The question arises whether the 'benzoin' sol has peculiar properties among lyophobe colloids that render it peculiarly liable to precipitation by such lyophile sols. Reference has already been made to the oil emulsions, investigated by Walpole. It may be noted that Gann found that acid gold sols were precipitated by gelatin. On the other hand, Eggerth and Bellows [1922] in a study of the agglutination of bacteria by various proteins in presence of buffered solutions of constant salt content and varying p_H showed that the amount of protein required varied with the p_H of the solution. In the case of 'benzoin' sol, however, the conditions are so simple that it seems particularly adapted for investigation of the principles of such precipitations.

The dependence of the protective power of gelatin on the isoelectric point is less marked than that of the precipitating power, though it is fairly clearly shown in Table XIII, and is minimum at p_H 4·7, the isoelectric point of gelatin. Apparently, ionised gelatin is better able to protect than isoelectric gelatin, and this may be correlated with the greater solubility of salts in water than the undissociated molecule. For the gelatin ions, either positively or negatively charged, will probably have a greater affinity for water than the undissociated uncharged molecules, just as the benzoate ion has a greater affinity for water and is, therefore, more soluble than uncharged benzoic acid. In this way, the result may be brought into line with the views of Langmuir on the affinity of certain groups for water, and of Loeb [1923]. Loeb shows that the protective effect of gelatin is to be ascribed largely to its high affinity for water, in fact, to its high true solubility.

The results we have obtained in regard to precipitation by salts support the view that such precipitation is largely a matter of the ion of charge opposite to that on the colloidal particle. The anomalies are to be, at least largely, explained as due to the varying p_H. This is but another example of the many instances in colloid chemistry where difficulties are cleared up if due note is made of the hydrogen ion concentration.

Conclusions.

1. Precipitation of colloidal gum 'benzoin' occurs when it is added to an equal volume of gelatin of concentration about 1/500,000. The actual concentration of gelatin required varies with the p_H of the solution.

2. No precipitation occurs on the alkaline side of the isoelectric point of gelatin.

3. The charge on the particles is negative if insufficient gelatin to precipitate it has been added. If excess of gelatin has been added so that precipitation does not occur, the charge is positive. On the alkaline side of the isoelectric point of gelatin the charge is always negative.

4. Similar results are obtained with oxyhaemoglobin.

5. The power of gelatin to protect 'benzoin' against precipitation by sodium chloride depends on the p_H and is minimum at the isoelectric point.

6. Precipitation by an acid depends essentially on the concentration of hydrogen ions.

7. The amount of salt required to precipitate 'benzoin' increases with increase in the p_H. This, at least largely, accounts for the differing amounts of salts of univalent kations required to effect precipitation.

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LXXXII. THE MECHANISM OF PRECIPITATION OF COLLOIDAL GUM BENZOIN BY CEREBROSPINAL FLUID.

By HEDLEY DUNCAN WRIGHT and WILLIAM OGILVY KERMACK.

From the Laboratory of the Royal College of Physicians, Edinburgh.

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In 1920 the use of a colloidal suspension of gum benzoin was introduced by Guillain, Laroche and Lechelle [1920] in order to detect certain pathological variations in the cerebrospinal fluid. The test is carried out by preparing in test-tubes 1 cc. of each of a series of dilutions of cerebrospinal fluid, 3/4, 1/2, 1/8, 1/16,...1/65,536, sixteen dilutions being used. To each tube is then added 1 cc. of a colloidal suspension of gum benzoin. In our experiments we have, in order to obtain uniform suspensions, slightly modified the original instructions given by the authors of the test, thereby obtaining a very uniform 'benzoin' sol, but one apparently rather less sensitive than the original. The sol used is fully described in the preceding paper.

Typical results obtained from fluids which are stated to give characteristic precipitations are as follows:

C.S.F. from case of General Paralysis of the Insane		344	443	444	310	000	0
,, ,, Tabes Dorsalis		003	410	241	000	000	0
,, ,, Meningitis (streptococcal)		000	001	444	443	100	0
,, ,, ,,		122	200	444	430	000	0
,, normal*	Between	000	000	242	000	000	0
	and	000	000	000	000	000	0

The system of indicating results is that described in the preceding paper.

* Fluids from absolutely normal individuals are naturally rarely obtained, and so 'normal' denotes fluid from patients where there is every reason to believe there is no essential change in the nature of the fluid, *e.g.* measles, dementia, epilepsy.

The main point to be noticed is that the precipitation occurs in two zones. There is a normal precipitation in the seventh or eighth tube, which in pathological fluids may or may not be increased. It is particularly increased in meningitis, and usually to a less extent in general paralysis; in fact, an extension of the zone to the right often accompanies an increase of protein in the fluid, as shown by the butyric acid test [Noguchi and Moore, 1909]. The first six tubes form another zone of precipitation. Here the precipitate is particularly marked in general paralysis, and it is also notably present in the case of tabetic fluids. That is to say, in syphilis of the central nervous system well-marked precipitation occurs in these tubes. There often is also slight precipitation in cases of meningitis, but in the normal fluid there is no precipitation at all. This zone, then, appears to be affected independently of the other.

Guillain, Laroche and Lechelle showed that neither the individual proteins (obtained by fractional saturation with ammonium sulphate) nor the dialysable constituents of a fluid could reproduce the curve obtained with the original fluid. A mixture of these could, however, do so. Dible [1922] suggests that the various curves are due to variation in cerebrospinal fluids in their globulin, albumin and salt content. In view of the fact that no account was taken by these workers of the actual p_H in the various tubes it is doubtful how these results should be interpreted. It may be noted that previous workers [Cruickshank, 1920; Lange, 1912] have shown that the portion of a cerebrospinal fluid which is most effective in precipitating a gold sol is that which is precipitated by half saturation with ammonium sulphate.

In the previous paper [1923] we have shown that the precipitation of 'benzoin' by gelatin and by oxyhaemoglobin depends very greatly on the hydrogen ion concentration of the medium. At a p_H on the alkaline side of the isoelectric point, no precipitation of the sol by gelatin takes place at any concentration of gelatin. At a p_H of about 4·7, precipitation takes place when 1 cc. of the sol is added to 1 cc. of gelatin diluted 1 in 640,000. As the p_H decreases, and the solution becomes more acid, the amount of gelatin required decreases, until at a p_H of 2·8 the hydrogen ions alone are sufficient to precipitate in the absence of gelatin, and precipitation occurs until a concentration of gelatin of at least 1/640,000 is present. Similar results have been obtained using haemoglobin. In view of the great dependence of the behaviour of proteins towards colloidal 'benzoin' on the reaction, a systematic investigation of the normal and paretic types of curves in this respect appeared to be called for. Accordingly, we have investigated the influence of reaction in the cases of serum globulin, serum albumin, and of whole serum, as well as of cerebrospinal fluids of the paretic and normal types on their behaviour as precipitants of colloidal 'benzoin.' The results explain the constancy of the zone of precipitation in the seventh and eighth tubes, and its variation in degree and not in position, and also demonstrate, we think, that the precipitation in the first five tubes (called by the authors of the test the syphilitic zone), is due, not to ordinary serum globulin or albumin, but to some extra material, which if it is protein in nature possesses a high isoelecric point, p_H between 7 and 8, and that in these cases, the actual serum globulin and albumin may be, at most, very slightly increased.

Experimental.

As small quantities of serum albumin and serum globulin apparently exist normally in the cerebrospinal fluid, these two proteins were first of all investigated. The mode of experiment was the same as described in the preceding paper. Fresh serum was fractionated by precipitation, first with half-saturated ammonium sulphate and then with saturated ammonium sulphate. The protein fractions obtained were washed well with ammonium sulphate solution of the requisite strength, and then transferred to a collodion sac, in which they were dialysed for 24–36 hours against tap-water. The albumin

solution was made up to the volume of the original serum and it was then ready for use. The globulin fraction was separated from the dialysed solution. It was filtered off and dissolved in sufficient sodium chloride solution (0·8 %) to make up the original bulk. If necessary it was filtered from some insoluble residue. Dilutions of these protein solutions were prepared, and also solutions of sodium hydroxide and of hydrochloric acid of various strengths. To a mixture of ½ cc. of a globulin solution and ½ cc. of acid or alkali was added 1 cc. of 'benzoin' sol. After shaking thoroughly, and standing overnight, the degree of precipitation was judged. The results are given in Table I, in which the dilutions given are those of the globulin or alkali after mixing, and before adding the 'benzoin.'

Table I.

Effect of p_H on precipitation by Globulin.

(The degree of precipitation is indicated by the same conventional numbers as in the preceding paper.)

Concentration of sodium hydroxide	Concentration of globulin solution: 1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120
N/800	0	0	0	0	0	0	0	0	0	0
N/1600	4 (p_H 5·3)	4	4	4	4	3	0	0	0	0
N/3200	0	0	0	0	0	4	1	0	0	0
N/6400	0	0	0	0	0	4	4	0	0	0
No alkali	0	0	0	0	0	2	4	2	0	0

It will be seen that with serum globulin a zone of precipitation occurs, which, just as in the case of gelatin and of haemoglobin, alters its position on addition of alkali. In the case of globulin the last row in which precipitation occurs has the p_H 5·3. A similar experiment using serum albumin shows no precipitation in solutions of p_H greater than 4·7. That is to say, these proteins do not cause precipitation in solutions the p_H of which is on the alkaline side of the isoelectric point, *i.e.* in solutions in which they exist as negative ions.

Precipitation of 'benzoin' by Serum.

Experiments were carried out to determine the effect of various dilutions of serum on 'benzoin.' The results are given in Table II.

Table II.

Precipitation by Serum.

The p_H given is that of the supernatant fluid. The charges on the colloidal particles are denoted by (+) or (−).

Concentration of serum	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
	0	0	0	0	0(−)	4 (p_H 5·4)	4 (p_H 4·7)	0(+)	0

Concentration of serum	1/1024	1/2048	1/4096	1/8192	1/16384	1/32768	1/65536	1/131072
	0	0	0(+)	4 (p_H 4·2)	4 (p_H 4·2)	2	0(−)	0(−)

It will be seen that there are two zones of precipitation—the first is at a concentration of serum 1/8192–1/16,384, or, assuming that the total protein content of serum is 7·5 %, about a concentration of protein of 1/130,000–

1/260,000. The charge on the 'benzoin' particles, as determined by the ultramicroscopic method described in the previous paper, is negative when the concentration of protein in solution is less than that corresponding to precipitation, and positive when it is greater. This zone then apparently corresponds to the normal zone of precipitation obtained with gelatin at concentration of about 1/600,000. The second zone of precipitation occurs at much higher concentrations of serum, 1/32–1/64. The charge on the particles is positive in tube seven and negative in tube four. The p_H of the supernatant fluid in tubes five and six is 5·4 and 4·7 respectively. It is obvious that here we are encountering, at the isoelectric point of the protein, the zone of precipitation which tends to broaden and to extend into regions of high concentration. In tubes one to four the p_H is above 5·4, and so no precipitation occurs and the particles are negatively charged. As soon, however, as the p_H falls below 5·4 precipitation occurs, as some of the protein molecules (globulin) now act as bases and are positively charged and tend to precipitate the 'benzoin.' When too much positively charged protein is present, protection occurs, because too much protein is absorbed, and so in tube seven there is no precipitation and the particles are positively charged. The commencement of the zone of precipitation naturally coincides with the isoelectric point of serum globulin (p_H 5·4) as this is higher than that of serum albumin (p_H 4·7). It would appear that, as the zone of precipitation extends from p_H 5·4 to 4·7, the presence of two proteins in concentrations of the same order of magnitude tends to produce a broad zone extending between the isoelectric points of the two proteins. This is probably to be explained on the consideration that, at a p_H between the two isoelectric points, much negatively, as well as some positively charged protein will be present. In that case there will be much less tendency for the particles of 'benzoin' to acquire a positive charge, although neutralisation may quite readily occur.

It is of interest to find that a somewhat similar phenomenon may be produced if gelatin be diluted 1 in 1000 in Ringer's solution and then diluted with an equal volume of distilled water in a series of successive tubes and 'benzoin' be added. The results of such an experiment will be found in Table III.

Table III.

Precipitation by Gelatin in Ringer's Solution.

Gelatin (1 part in 1000 parts of Ringer-Tyrode solution) was prepared, and this was diluted with distilled water to the dilutions given, an equal volume of 'benzoin' sol was added to each dilution and the results read after standing overnight.

Concentration of gelatin × 1000	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
	0	0	0	0(−)	0(−)	1	0(+)	2	4	4	3½

Here two zones of precipitation appear, the first corresponding with a change in the charge on the particles from negative in high concentration to positive in low, the second with a change from positive in high concentration to negative in low. It is to be noted that Ringer's solution alone has no precipitating

effect on 'benzoin,' notwithstanding its comparatively high concentration of salt. This is because its p_H is 7·6.

Precipitation of 'benzoin' by Normal Cerebrospinal Fluid.

A normal cerebrospinal fluid was examined in regard to precipitation of 'benzoin' and the charges on the particles in the unprecipitated zones. The curve obtained was as follows:

000, 000, 320, 000, 000, 0.

Precipitation of about the usual amount was seen in the seventh and eighth tubes. On investigating the reaction in tube 7 this was found to be equivalent to p_H 4·5. It was, however, interesting to find that the charges on the particles of 'benzoin' were negative on either side of the zone of precipitation. The explanation is evident from the following experiment which shows the effect of the addition of acid or alkali to another normal fluid.

Table IV.

Effect of p_H on precipitation by normal C.S.F.

½ cc. of diluted C.S.F. and of acid or alkali added to each tube and then 1 cc. of 'benzoin' solution. Reading after standing overnight.

		Concentration of Cerebrospinal Fluid (Normal)											
		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Concentration of sodium hydroxide	N/400	0	0	0	—	—	—	—	—	—	—	—	—
	N/800	0	0	0	0	—	—	—	—	—	—	—	—
	N/1600	0	0	0	0	0	0	—	—	—	—	—	—
	N/6400	0	0	0	0	0	0	0	0	—	—	—	—
Distilled water		0	0	0	0	0	1	0	0	0	0	—	—
Concentration of hydrochloric acid	N/6400	0	0	0	0	0	3 (p_H 4·4)	1	0	0 (−)	0	0	0
	N/1600	0	0	0	0	4 (p_H 4·6)	4	4 (p_H 3·4)	1	0	0	0	0
	N/800	0	0	0 (−)	4 (p_H 4·7)	4 (p_H 3·4)	4 (p_H 3·3)	4	4 (p_H 3·0)	3	3	2	0
	N/400	0	0 (−)	4 (p_H 4·7)	0 (+)	0	4 (p_H 3)	4 (p_H 3)	4 (p_H 2·8)	4	4	4	4

Fig. 1 shows the resulting zone of precipitation if the concentration of fluid be plotted against the p_H of the tubes showing precipitation.

Here it will be seen that the added acid or alkali had a marked effect on the position of the zone of precipitation. This zone varies in a similar fashion to a zone of precipitation obtained with a protein. It does not extend to tubes which have a p_H greater than 4·7. This corresponds to the isoelectric point of serum albumin.

It will be evident from the table and figure that in a normal C.S.F. test, the zone of precipitation is not really crossed at all. It is entered and left again, as shown by the dotted line in the figure, which indicates approximately the p_H of the liquid in the various tubes after the 'benzoin' has been added, but where neither acid nor alkali is added. The result is that the 'benzoin' particles on both sides of the zone of precipitation are negatively charged since the positive region is never entered.

The reason for the constancy of position of the zones of precipitation obtained with normal fluids is evident. In the tubes in the first column, the amount of C.S.F. present is high and so there is considerable buffering of the

solution. The result is that after addition of the 'benzoin' the p_H remains high and it is not until the seventh or eighth tube that the acidity of the 'benzoin' is sufficient to lower the p_H to about 4·7. At this point the protein will have

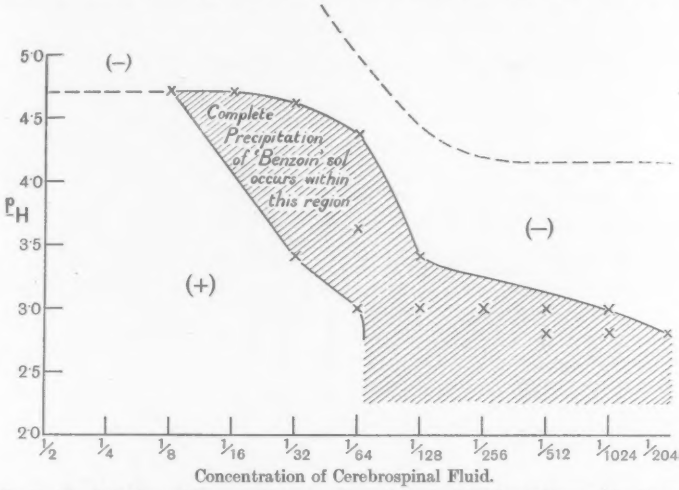

Fig. 1. Precipitation of 'benzoin' by normal cerebrospinal fluid as influenced by the p_H. The dotted curve represents approximately the actual p_H of the tubes to which no acid or alkali is added. As will be seen, the region of complete precipitation is not entered, the maximum precipitation being of the order 1/2. In other cases the line may enter and leave but does not cross the zone of complete precipitation. (+) and (−) refer to the charge on the 'benzoin' particles in the different regions.

lost its negative charge and so will begin to act as a precipitant of the 'benzoin.' Naturally, owing to slight variations in amount of buffering of the solution (CO_2 content, etc.), and possibly in the amount of protein the exact p_H in the seventh and eighth tubes will vary slightly; hence the slight variations in the amount of precipitation produced at this point even with normal cerebrospinal fluids. As we have remarked above, with some there is no precipitation at all. It is clear that this will occur where the protein content is low, or the degree of buffering is high. On the other hand, it is clear that in fluids, *e.g.* from a case of meningitis, where the protein content is much in excess, the zone will be extended into regions of higher dilution.

Precipitation of 'Benzoin' by a Cerebrospinal Fluid from a case of General Paralysis.

In the following experiment to determine the effect of acid and alkali on the zone of precipitation, a typical fluid from a case of general paralysis giving the following curve was used:

444, 443, 443, 100, 000, 0.

The essential point of difference between this and the normal curve is that complete precipitation has occurred in the first five tubes,

The details of the experiment recorded are the same as in the case of the normal fluid.

The result is shown in Table V.

Table V.

Effect of p_H on precipitation by Paretic C.S.F.

Concentration of cerebrospinal fluid (G.P.I.).												
		1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Concentration of sodium hydroxide	*N*/100	—	0	0	0	0	0	0	0	0	0	0
	N/200	—	0	0	0	0	0	0	0	0	0	0
	N/400	3 (p_H 7·8)	0	0	0	0	0	0	0	0	0	0
	N/800	4 (p_H 7·6)	0	0	0	0	0	0	0	0	0	0
	N/1600	4 (p_H 7·8)	4 (p_H 7·5)	3	0	0	0	0	0	0	0	0
	N/12800	4 (p_H 7·6)	4 (p_H 7·2)	4 (p_H 6·8)	0 (−)	4 (p_H 5·1)	2	0	0	0	0	0
Distilled water		4 (p_H 7·3)	4 (p_H 7)	4 (p_H 6·8)	3	4 (p_H 5)	3 (p_H 4·4)	0	0	0	0	0
Concentration of hydrochloric acid	*N*/6400	—	4 (p_H 7·2)	4 (p_H 7)	4 (p_H 5)	4 (p_H 4·6)	4 (p_H 4·1)	3	0	0	0	0
	N/1600	—	4 (p_H 7)	4 (p_H 6·6)	0 (+)	0	4 (p_H 3·8)	4 (p_H 3·6)	3 (p_H 3·4)	0	0	0
	N/1200	4 (p_H 7·4)	4 (p_H 6·9)	4 (p_H 5·4)	—	—	—	—	—	—	—	—
	N/800	4	4 (p_H 6·9)	0	—	—	—	—	—	—	—	—
	N/600	4	4 (p_H 6·2)	0	—	—	—	—	—	—	—	—
	N/400	4 (p_H 7)	4 (p_H 5·5)	0	0	0	0 (+)	4 (p_H 3)	4	4	4	4
	N/300	4 (p_H 7)	0	0	—	—	—	—	—	—	—	—
	N/200	4 (p_H 5·9)	0	0	0	0	0	4 (p_H 2·6)	4	4	4	4
	N/100	0	0	0	0	0	0	4 (p_H 2·4)	4	4	4	4

Fig. 2 shows the zone of precipitation if the p_H be plotted against the concentration of fluid in tubes showing precipitation.

It will be seen that up to a p_H of about 5 the curve is very similar to that given by a normal fluid. Notwithstanding the increase in total protein content, as shown by the Noguchi reaction, the zone of precipitation at p_H 4 occurs at a concentration of about 1/64 just as with the normal fluid. It would appear from this that there is no definite increase in the amount of serum globulin or albumin present, because if this were increased one would expect that the zone would be shifted to the right. On the other hand, precipitation is seen to occur in a region between p_H 5 and p_H 7 to 8. This is a zone non-existent with a normal fluid, serum globulin or serum albumin and so presumably the substance which causes this precipitation is not present in these.

If it is a protein, and the marked increase in the Noguchi reaction in most cases of general paralysis might suggest this, it is presumably a protein of isoelectric point p_H 8, since the 'benzoin' is negatively charged and proteins, as far as our experiments go, precipitate it only when they are at, or on the acid side of, their isoelectric point. They fail to do so when they are negatively charged. At p_H 7 the normal proteins (serum albumin and serum globulin) are negatively charged and it is difficult to understand how they could be the active agents.

It may be noted that precipitation occurs over a continuous area. This might at first sight appear rather inexplicable, as instead two zones might rather be expected. However, the explanation is, no doubt, similar to that of the existence of a zone with normal serum extending approximately from

p_H 5·4 to 4·7, that is, between the isoelectric points of serum globulin and of serum albumin. If two proteins are present in comparable concentrations, then at a p_H between the two isoelectric points, the positively charged particles of the protein with the higher isoelectric point will neutralise the negative

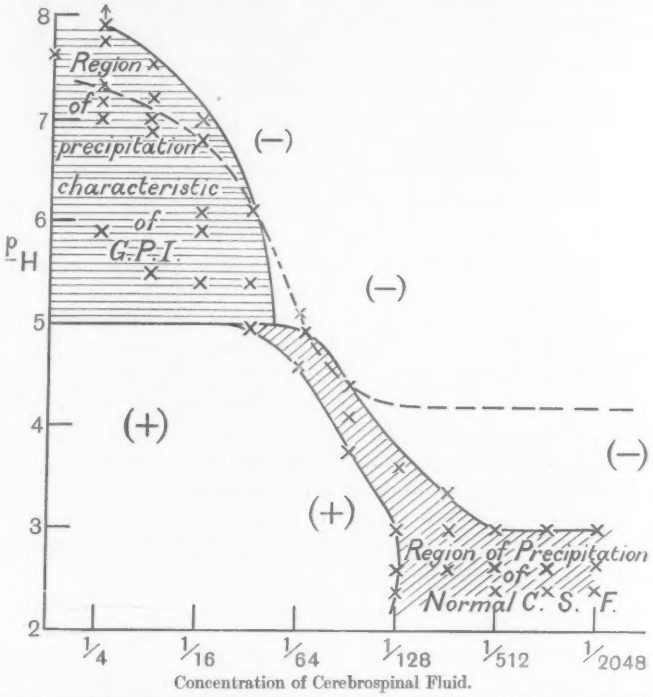

Fig. 2. Precipitation of 'benzoin' by cerebrospinal fluid from patient with G.P.I. The dotted curve represents approximately the actual p_H of the tubes to which no acid or alkali is added.
(+) and (−) refer to the change in the 'benzoin' particles.

charge on the colloidal 'benzoin' particles, but it is doubtful whether it will confer a positive change on them, as the other protein in solution is still completely negative, and so will tend to prevent the positive protein being absorbed on the particles.

Discussion.

Goebel [1921] has made the observation that in the case of the mastic reaction, on diluting cerebrospinal fluid from a case of general paralysis with a normal fluid, the curves obtained are still essentially of the paretic type. He further suggests that there are two fundamental types of curve—the meningeal type indicating increased permeability of the vessels and consequent increase in serum protein in the fluid, and the paretic type characteristic of conditions associated with tissue destruction. Lange [1912] would appear

to suggest that in paretic fluids evidence of the presence of nucleoprotein may be obtained. Cruickshank [1920] suggests that in general paralysis there is a change in the ratio of globulin to albumin in the cerebrospinal fluid and as the globulins from a normal fluid do not give the reaction even on concentration, this is associated with an alteration in the electrical charge on the globulins.

Our own results confirm the view that there is a qualitative difference between the paretic cerebrospinal fluid and the normal, as in the table showing precipitation by a paretic fluid the zone due to the normal constituents can be identified and differentiated from the peculiar and characteristic abnormal zone. The essential feature of the latter is that it occurs between p_H 5·5 and 7·5, and it is clear that no interpretation or explanation of a curve can be made without taking into account the reaction of the solution in which precipitation occurs. This, of course, is in accordance with the fact that the physico-chemical behaviour of proteins is a function of the reaction, more particularly of the reaction relative to their isoelectric point.

The general effect of alkalinity on the colloidal reactions of the cerebrospinal fluid has already been noted in the literature. For instance, Bannerman [1919] has shown that the globulin fraction of a paretic fluid can reproduce the paretic curve of gold, if it is made slightly alkaline, and Presser and Weintraub [1922] and Sahlgren [1922] attributed the non-precipitation of mastic by high concentrations of normal cerebrospinal fluid to the protective action of the alkali present. But the precipitation of a colloid is determined by the two factors protein and reaction together, and these cannot be considered independently, for it is the electrical condition of the protein that is of importance.

Conclusions.

1. The zone of precipitation occurring in normal and pathological cerebrospinal fluids in the seventh and eighth tubes in the colloidal 'benzoin' test is due to precipitation of the negatively charged 'benzoin' particle by the positively charged protein of the fluid.

2. The constancy of its position depends on the fact that the p_H must be below 4·7 before any such precipitation can occur. The variations in the degree of precipitation and in the number of the tubes included in the zone, depends on the relative amount of protein present and the degree of buffering of the fluid.

3. The precipitation occurring in the first five tubes in fluids from cases of general paralysis of the insane is due to the presence of some abnormal constituent, and not to an increased amount of the proteins responsible for the normal precipitation.

4. The general dependence of precipitation by cerebrospinal fluid on the hydrogen ion concentration has been investigated.

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LXXXIII. OBSERVATIONS ON THE SOLUBILITY OF INSULIN.

By ERIK MATTEO PROCHET WIDMARK.

From the Institute of Medical Chemistry, Lund, Sweden.

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It is of the greatest importance for the isolation and purification of insulin to know its solubility in different organic solvents. Our knowledge as to this has been up till now very limited. Insulin is freely soluble in water. We know, further, that it is insoluble in ethyl ether, toluene and neutral ethyl alcohol. Doisy, Somogyi and Shaffer [1922] find that insulin is certainly soluble in water, but that it is incompletely precipitated from its solution at p_H about 5 to 6. They find, also, that it is soluble in "alcohol" except at its isoelectric point.

The following experiments on the solubility of insulin have been performed with "crude insulin" prepared partly according to Collip [1922] and partly according to Shaffer. About 20–25 mg. contained one "rabbit-unit" and 0·04–0·02 mg. produced characteristic convulsions in mice.

The solubility experiments were made in the following way. It might be expected that the salt of insulin would have a different solubility from the free compound. For this reason we have made the experiments in two different series: acid and alkaline extractions.

Extractions at acid reaction. 10 mg. insulin were dissolved in 1 cc. water and 1 cc. acid buffer solution (equal parts of *N* acetic acid and *N* Na-acetate, p_H about 4·75) was added. The solution was equally distributed (0·2 cc.) in ten small Petri dishes and evaporated to dryness in a vacuum exsiccator over sulphuric acid. The activity of the insulin was not hereby diminished. 2–3 cc. of the organic solvent were now added. The solvent was filtered from the residuum and again evaporated in a dish. 2 cc. of physiological salt solution were added and 0·5 cc. of this solution was injected into each mouse. The animals were placed in a thermostat at 28° and the occurrence of convulsions was noted. The convulsions demonstrated the solubility of the insulin in the solvent used. The activity of the residuum from the extractions was often controlled in the same way in order to show that the insulin had not been destroyed during the operations.

The extraction at alkaline reaction was made in the same way only with the difference that an alkaline buffer was used (sodium hydroxide-borate, p_H 10) instead of the acid.

With this method we tried some different organic liquids until we found in absolute methyl alcohol the first water-free solvent.

Extraction at acid reaction.

Solvent	Extract	Residuum
Tetrachloromethane	− − − −	+ + + +
Ethyl acetate	− − −	+ + − −
Light petroleum	− − −	+ −
Methyl alcohol	+ +(+)	+ + −

The plus sign indicates that convulsions occurred, the minus sign that they failed to appear. With tetrachloromethane, ethyl acetate and light petroleum no effect on the animals could be observed. This means that, inasmuch as the quantity of insulin used was many times greater than that which produces convulsions, the insulin is insoluble or only very slightly soluble in the solvents mentioned. The methyl alcohol on the contrary dissolves the insulin, although the extraction is incomplete.

Extraction at alkaline reaction.

Solvent	Extract	Residuum
Tetrachloromethane	− − −	+ + +
Ethyl acetate	− − −	
Ethyl ether	− − −	
Ethyl alcohol	− − −	
Chloroform	− − −	
Acetone	− − −	
Light petroleum B.P. below 50°	− − −	
„ B.P. 60–80°	− − −	
Methyl alcohol	+ +(+)	+ + +

Here, too, we obtain the same results. Methyl alcohol alone is able to extract the insulin.

The solubility experiments with methyl alcohol were repeated on a larger scale. Neutral, anhydrous methyl alcohol dissolves the insulin only with difficulty. Acid methyl alcohol dissolves it much more easily. The pure alcohol was acidified by passing in dry HCl. 200 cc. of this liquid, containing 6·6 millimols HCl dissolved 500 mg. crude insulin only leaving a slight residue.

The insulin may be precipitated from the methyl alcoholic solution by reagents which mix with methyl alcohol, but in which the insulin itself is insoluble. This proceedure was used in order to detect other solvents for insulin. A saturated solution of insulin in pure, neutral methyl alcohol was prepared. The solution was distributed in a series of test-tubes and several volumes of the different fluids were added to each of the tubes. A precipitation occurred with the following fluids: ethyl acetate, ethyl isobutyl, and amyl alcohol, chloroform, tetrachloromethane, acetone, light petroleum, ethyl ether, benzene, xylene and pyridine. Whether the precipitates contained insulin was tested by activity experiments on mice, positive results being obtained with those from ethyl acetate, ethyl, isobutyl, and amyl alcohol, ethyl ether, light petroleum, acetone and tetrachloromethane. These experiments thus show the insolubility of the insulin in these solvents.

By the addition of glacial acetic acid, phenol, formamide and glycerol to the methyl alcoholic solution no precipitations were obtained. The solubility in glycerol can be explained through the high water content of this solvent.

But anhydrous glacial acetic acid, phenol and especially formamide were found capable of dissolving considerable amounts of insulin. It could be precipitated from these solutions, for instance, by ethyl ether, and this precipitate was very active.

These facts concerning the solubility of the insulin may be of value for further research on the nature of this hormone. They do not say much about its chemical nature, but I will remark that methyl alcohol, phenol and above all formamide are solvents for albumoses. Other facts also favour the belief that insulin belongs to this class:

1. Crude insulin gives a decidedly purple colour by the biuret test, and an orange colour by the xanthoproteic test.
2. It gives a precipitate with nitric acid, which disappears on heating.
3. It is not precipitated on boiling.
4. It is precipitated from watery solution by half saturation with ammonium sulphate.
5. It is very easily destroyed by trypsin.
6. It is impossible to demonstrate any dialysis of insulin through parchment membranes. It cannot be ultrafiltered through 3 % collodion filters.
7. It is freely soluble in pure, salt-free water.

I cannot find any valid reason for the suggestion that insulin is a globulin.

SUMMARY.

1. Insulin is insoluble or very slightly soluble in tetrachloromethane, ethyl acetate, ethyl alcohol, isobutyl alcohol, amyl alcohol, chloroform, acetone, light petroleum, ethyl ether, benzene, xylene and pyridine. Easily soluble in methyl alcohol, glacial acetic acid, phenol and formamide.
2. Several facts argue in favour of its being an albumose.

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